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FINAL REPORT

VIDO - FIVE YEAR RESEARCH PROGRAM II

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Adding value to agriculture...through research and development

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Saskatchewan Agriculture Development Fund-VIDO

Contract Final Report

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EXECUTIVE SUMMARY

At the start of the funding period, VIDO was engaged in 11 research projects dealing with the development of novel vaccines and vaccination technologies for use in livestock and poultry. A list of the specific projects is shown below. Projects spanned the beef, swine, dairy and poultry industries and dealt with the development of both bacterial and viral vaccines plus new technologies for vaccine formulation and delivery which are applicable to all sectors. Our specific projects were as follows:

1. Disease-specific Vaccines
   a) Bovine respiratory disease (*Haemophilus somnus* and bovine herpesvirus-1)
   b) Bovine mastitis
   c) *Streptococcus suis* infection in swine
   d) *Escherichia coli* infection in poultry
   e) Bovine rotavirus and coronavirus (calf scours)
2. Vaccination technologies
   a) Live adenovirus vaccine vectors
   b) Nucleic acid immunization
   c) Immune responses at mucosal surfaces
   d) Vaccine formulation and delivery
3. Diagnosis
   a) Measurement of host status

The disease-specific vaccine projects included those in early stages of development (eg. bovine mastitis) as well as those for which proof of concept had been demonstrated (eg. bovine respiratory disease). The latter technologies were based upon research conducted during the early 1990s and much of the activities were related to technology transfer. In all cases, proof of concept for vaccination as a control measure has been demonstrated, with the exception of *Escherichia coli* vaccines for poultry. The vaccine formulation and delivery projects build upon expertise in immunology developed during the 1980s and 1990s and addressed a critical need in the field of vaccinology. These projects are viewed as platform technologies which will be applied to all current and future vaccine-specific development projects at VIDO.

Vaccine-specific Projects

Over the past five years, significant progress has been made in all project areas. In the case of the bovine respiratory disease vaccines, we have transferred the technology for recombinant subunit vaccines to the private sector for commercial development as has been described in previous reports. Unfortunately, due to changes in the animal health industry over the past five years, our initial development partner, BIOSTAR Inc. no longer exists and thus the technology for bovine herpesvirus-1 vaccines has been transferred to Novartis Animal Health. In the case of *H. somnus*, the technology for vaccine production was transferred to Bayer Animal Health and after their restructuring, it was transferred to the Alberta Research Council for scale-up and licensing. The technology for the bovine rotavirus and coronavirus vaccine has been successfully tested under field conditions and a licensing agreement reached with Vetrepharm Animal Health, a Canadian

veterinary vaccine manufacturer. We have continued to co-develop this vaccine with Vetrepharm Animal Health and it is now at the stage where a submission is being made to the Canadian Food Inspection Agency for licensing. This particular vaccine has several advantages over the product developed at VIDO during the 1980s, including broader spectrum protection due to the inclusion of antigens from other rotavirus serotypes which are now prevalent in Canada as well as a simplified production protocol. Thus, the research phase on all three of the above projects is complete and VIDO is no longer actively working on them other than specific contracts in collaboration with our commercial partners.

The *Streptococcus suis* and bovine mastitis vaccine projects were in their early development phases at the start of this funding period and in both cases, we have now demonstrated proof of concept for vaccination as a control method for these diseases. In the case of *Streptococcus suis*, we attempted to develop a recombinant subunit vaccine but quickly found that a conventional approach was more effective. There are over 30 different serotypes of *S. suis* and the identification of single antigens which would cross protect against all of these was not deemed to be possible at the current time. However, we also developed conventional vaccine technologies which have now been transferred to the Alberta Research Council for scale-up and licensing. In the case of bovine mastitis, our objective was to identify vaccine components from the Streptococcal species associated with this disease. This included *S. uberis*, *S. dysgalactiae* and *S. agalactiae*. Over the past five years, we have successfully identified three classes of antigens which are present in each species and have tested their vaccine potential in experimental models. In the case of *S. uberis* and *S. dysgalactiae*, excellent protection against experimental infection has now been demonstrated whereas for *S. agalactiae*, the protection was not as good. However, the first two organisms account for the bulk of Streptococcal mastitis cases in the field we are therefore proceeding with further development based on these three groups of antigens. During the past two years, we have focused on the construction of chimeric antigens in order to reduce the production cost for this vaccine. Proof of concept for the use of these antigens has been demonstrated and during the past year, we signed a research and development agreement with a multinational animal health company to further develop this vaccine and ultimately license it for use internationally. We anticipate that the research phase of this project will be complete over the next 12-24 months.

The final vaccine project dealt with *Escherichia coli* infection in poultry. This organism causes a number of different disease syndromes in both chickens and turkeys, including infections of the heart, liver, respiratory tract and skin. Our approach to the development of *E. coli* vaccines was to cripple the bacteria through the introduction of specific lesions in their DNA so that they were no longer able to cause disease. These attenuated strains were then delivered orally to birds via drinking water or by aerosol. We were able to successfully demonstrate proof of concept for immunization of turkeys using this vaccine. However, over the past five years, cellulitis in chickens has become the predominant disease manifestation and our research has indicated that it is not possible to prevent cellulitis by vaccination. Thus, an avian *E. coli* vaccine would have limited utility in the field. Therefore, during the past year, we ceased work on this project after consultation with the poultry industry and have initiated a new project on the development of *Campylobacter jejuni* vaccines which will prevent colonization. While this organism does not cause disease in poultry, it is a significant human pathogen that can be transmitted via contaminated meat.

Vaccine Formulation and Delivery

Despite advances made in the identification and production of specific protective vaccine components, most vaccines are still being formulated and delivered using technologies that are over a century old. The majority of products available for use in animals are mixed with oil-based adjuvants to increase the immune response and are injected either subcutaneously or via the intramuscular route. The use of oil-based adjuvants has been raised as a concern due to the presence of oil residues in meat products. Also, injection of such products has been shown to cause significant economic losses to producers due to tissue damage at injection sites. Thus, we have focused on the development of new technologies for the formulation and delivery of vaccines, including novel subunit vaccine formulations as well as the use of animal viruses to deliver heterologous antigens. Both of these technologies have the advantage of stimulating the appropriate type of immunity at the appropriate site within the animal, resulting in the possibility of more efficacious vaccines which are safer to use. Over the past five years, VIDO has successfully demonstrated that animal adenoviruses can be used to deliver antigens from a variety of pathogenic microorganisms. We have identified sites within bovine and porcine adenovirus which can be deleted and have inserted a variety of genes coding for viral glycoproteins. The use of these recombinant viruses as vaccines has been demonstrated using both bovine respiratory disease and bovine enteric models. In the case of bovine adenovirus vectors, there has also been considerable interest in applying this technology to the field of human gene therapy. Over the past five years, we have collaborated with a European gene therapy company on this aspect of the technology and are now pursuing this work further with a North American company. While there are still significant regulatory questions which must be addressed before the technology will find use in the field, we are confident that our partnership with the private sector will result in products being available to producers in the near future.

As was mentioned above, most subunit vaccines require adjuvants in order to elicit effective immune responses. We have been developing alternatives to oil-based adjuvants, including liposomes, through a collaboration with researchers at the University of Saskatchewan's College of Pharmacy. We have successfully demonstrated that when vaccines are formulated with liposomes, they can be delivered via a variety of routes (intranasal, transcutaneous, intradermal, subcutaneous) with significant increases in respiratory immunity compared to traditional vaccine formulations. We have demonstrated this with both animal and human vaccines. These particular products also have the advantage of allowing one to include biological response modifiers with the antigens to direct the immune response to that appropriate for the specific disease. We have integrated the use of these delivery technologies into the vaccine-specific projects described above and believe that the combination will result in novel products for the industry which are more efficacious and safer to use. For example, we are testing liposome-based intranasal mastitis vaccine formulations and the results to date indicate that these are more efficacious than conventional formulations.

Most of the organisms which VIDO currently works on cause disease within the respiratory or digestive tracts. These mucosal surfaces utilize a different arm of the immune system than that which is stimulated by intramuscular or subcutaneous vaccination and we believe that more effective products can be developed by targeting vaccines to these specific sites in the animal. The Immunology Group at VIDO has developed new models to study mucosal immune responses and utilize these to test vaccine formulations delivered via intranasal and oral routes. In the latter case,

we are collaborating with a multinational animal health company on the use of alginate microspheres which can be used to encapsulate a variety of vaccine types, including live attenuated adenoviruses as well as subunits. The use of such formulations should enhance the efficacy of vaccines delivered orally by protecting the antigens from degradation in the digestive tract of animals. These models have also been used to demonstrate a novel strategy for vaccination, namely immunization of the fetus. The rationale behind this particular technology is to stimulate immunity prior to birth of the animal (or human) which will enhance protection against disease in both the neonate and throughout the lifetime of the animal. VIDO's work in this area is the first time that this particular technology has been successfully demonstrated in an animal species.

All of the technologies described above deal with the development of either subunit or live vaccines. One of the problems with utilizing such technologies for viral products is the high cost of production of such antigens. One method of circumventing this problem is to immunize with the genes coding for protective antigens. These genes can be propagated and isolated in large quantities using bacterial production systems, reducing the cost of vaccine production. VIDO was the first group to demonstrate proof of principle for DNA immunization in the target species and we have continued to develop these specific vaccines for use in livestock. Our research has highlighted the need for new delivery systems and adjuvants which can be used with DNA-based vaccines and thus we have integrated projects dealing with the use of specific oligonucleotides as adjuvants. In addition, the delivery technologies described above (liposomes, microspheres) are being tested with nucleic acid vaccines as well as their subunit counterparts. We have also pursued alternative delivery strategies utilizing "gene guns" with DNA vaccines and have demonstrated proof of principle in this area also. We are pursuing the use of such delivery devices with a number of pharmaceutical companies.

Future Directions

VIDO's research projects have historically been in high risk areas and have been targeted at the development of technologies and products which are not being developed elsewhere. For example, during the 1980s our focus was in the area of biotechnology and its application to veterinary vaccine production and it is only in the past five years that this particular technology has finally been accepted as the norm by the animal health industry. As we move into the next millennium, it is clear that the area of genomics will provide the same opportunities as biotechnology during the 1980s and 1990s. VIDO has been active in this area for over a decade and indeed, we currently hold patents on entire genomic sequences of veterinary pathogens. However, we will expand our activities in this area, especially for new research projects as they come on stream. For example, our new project dealing with *Campylobacter jejuni* vaccines will utilize a strictly genomic approach to vaccine development. These activities will be enhanced by the current expansion underway at VIDO as well as through collaborations and partnerships with other institutions. We are also moving into non traditional areas of vaccine development such as vaccines for food safety pathogens. These are microorganisms which do not cause disease in animals but can be transmitted to humans via animal products. We are in the final stages of testing an *E. coli* O157:H7 vaccine for cattle in partnership with the University of British Columbia and anticipate including *Salmonella enteriditis* vaccines for poultry as well. The latter will be combined with the *Campylobacter jejuni* vaccine project.

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1. VIDO'S MANDATE

VIDO's mandate is to serve the Canadian livestock and poultry industry by conducting animal health-related research, communicating livestock management techniques and information, and facilitating the transfer of technology for international commercial development.

VIDO's Goals

- To serve and assist the economic competitiveness of the livestock industry through research on the common infectious diseases of animals and poultry.
- To provide information leading to safe and effective animal health preventive medicine programs which enhance animal care through improved management and performance of livestock.
- To maximize funding by enhanced visibility and development of innovative communication programs with all organizations that provide support to VIDO.
- To transfer technology to the biology industry to enhance its commercial application for the benefit of the Canadian livestock producers and to provide financial stability to VIDO.
- To identify opportunities to utilize VIDO's livestock research to improve human and companion animal health.
- To manage its financial, educational, and human resource efforts to ensure long-term benefits to the organization's stakeholders.

2. VIDO'S RESEARCH PROGRAMS

1. Pathogenesis and control of *Streptococcus suis* infection in swine
2. *Escherichia coli* infection in poultry
3. Control of bovine mastitis
4. Subunit vaccines for *Haemophilus somnus* **(complete)**
5. Vaccines for bovine rotavirus and coronavirus **(complete)**
6. Vaccines for bovine herpesvirus-1 **(complete)**
7. Development of bovine adenovirus as a vaccine vector
8. DNA immunization
9. Measurement of host status **(complete)**
10. Development of models for the evaluation of mucosal immune responses
11. Vaccine formulation and delivery

3. RESEARCH ACCOMPLISHMENTS

3.1 Project Title: Pathogenesis and Control of *S. suis* infection in swine.

3.1.1 Objective:

Our objective is to develop methods for reducing economic losses in swine production due to *Streptococcus suis* infection by describing the epidemiology of the disease and developing novel subunit vaccines.

1. Cloning and expression of genes coding for antigenic proteins
2. Produce immunological reagents in rabbits
3. Initiate vaccine trials using recombinant proteins
4. Complete the cloning and expression of *S. suis* genes
5. Determine protein expression during infection
6. Develop ELISA for porcine antibody to specific antigens
7. Complete vaccine trials with recombinant proteins using homologous challenge
8. Determine the cross-serotype protective capacity of recombinant proteins

3.1.2 Background and Approach:

Streptococcus suis cause a variety of disease syndromes in swine, including septicemia and meningitis, which were identified by the swine industry as a priority infectious disease problem for VIDO to address. Our approach was to first identify the extent of the problem in Western Canadian swine herds by determining the prevalence of infection of pigs with *S. suis*, the prevalence of disease and the different types (serogroups) of bacteria present in the population. This necessitated the development of a diagnostic test to measure serum antibody levels to the bacteria. Once the extent of the problem had been determined, we tested the feasibility of vaccination as a control measure by producing a special kind of conventional subunit vaccine. This vaccine, designed to induce immunity to bacterial factors responsible for disease, was shown to be responsible for significant protection in the offspring of vaccinated sows. Since it may not be economical to produce this special kind of conventional vaccine on a commercial basis, we focused on the identification of the protective components of this vaccine and evaluated antigen production using recombinant DNA technology to produce large quantities of protein in a cost-effective manner.

- Most swine herds in Canada have pigs that are infected with *S. suis*.
- Infection occurs with intermittent disease or without disease in many herds.
- Whether or not disease occurs may be due to environmental and management factors in addition to bacterial virulence factors and pig immunity.
- We have shown that vaccination to increase pig immunity and control disease losses is feasible, but an effective, economical vaccine is not currently available.

3.1.3 Progress:

a) Cloning and expression of genes coding for antigenic proteins.

We have identified antigenic *S. suis* proteins, produced antisera to them and identified recombinant DNA clones that produce some of these proteins. Specific immune serum produced in rabbits 71/72, was used to screen our recombinant library of *S. suis* genes in *E. coli*. We have

identified 52 clones that reacted with this serum in a colony blot assay. Subsequent analysis of total cellular proteins from these colonies using Western Blot technique showed that most of them produced a protein of approximately 44 kDa molecular weight that reacted with the 71/72 rabbit serum. Restriction endonuclease analysis of the plasmid DNA in these clones also confirmed that they were genetically similar. One of these clones (203/449#10) was selected for further study.

As indicated in the table below, 5 of the 6 identified genes have been cloned and expressed in various amounts. Note that the gene for the 80 kDa protein has been cloned in two forms to facilitate expression and purification. We have produced monospecific rabbit antiserum to each of the 5 proteins and shown that strains of *S. suis* representing 18 serotypes react with each of these sera in western blot reactions.

Protein ID	Size (kDa)	DNA Cloning Status
Pyruvate Kinase (truncated)	35	Cloned and expressed
Phosphoglycerate Kinase	44	Cloning in Progress
Phenylalanine Synthase	44	Cloned and expressed
Ornathine Carbamoyl Transferase	47	Cloned and expressed
Streptococcal Acidic Glycoprotein	51	Cloned and expressed
80 kDa Protein (fusion with glutamyl amino peptidase [GAP])	80	Cloned and expressed
80 kDa Protein (fusion with histidine tag)	80	Cloned and expressed

b) Produce immunological reagents in rabbits.

We have produced specific rabbit sera (#96-01/02) that were used to specifically identify *S. suis* (Serotype 2) bacteria in formalin-fixed swine tissue. In meeting this objective, we have produced a tool that can be used to identify *S. suis* bacteria in fixed tissue. The advantages of this method include the ability to identify killed bacteria which cannot be grown in the laboratory, for retrospective studies using stored, fixed material, and for clearly showing the pathogenic role of these bacteria by revealing them in the context of histopathologic lesions in brain tissue. These sere were produced by exposing rabbits to intact *S. suis* bacteria and much of the reaction is with polysaccharide surface antigens.

One additional goal was to use protein-specific antisera in this technique. We have produced 1086-specific rabbit anti-sera (#97-26/27) and SAGP-specific rabbit anti-sera (#97-30/31) to detect expression of these *S. suis* proteins in the brain.

Unfortunately, these sera do not react with *S. suis* in formalin-fixed brain tissue. Studies are in progress to determine whether the lack of reactivity is due to modification of protein antigen epitopes that occurs during formalin-fixation or due to masking of these antigens by other host or bacterial material

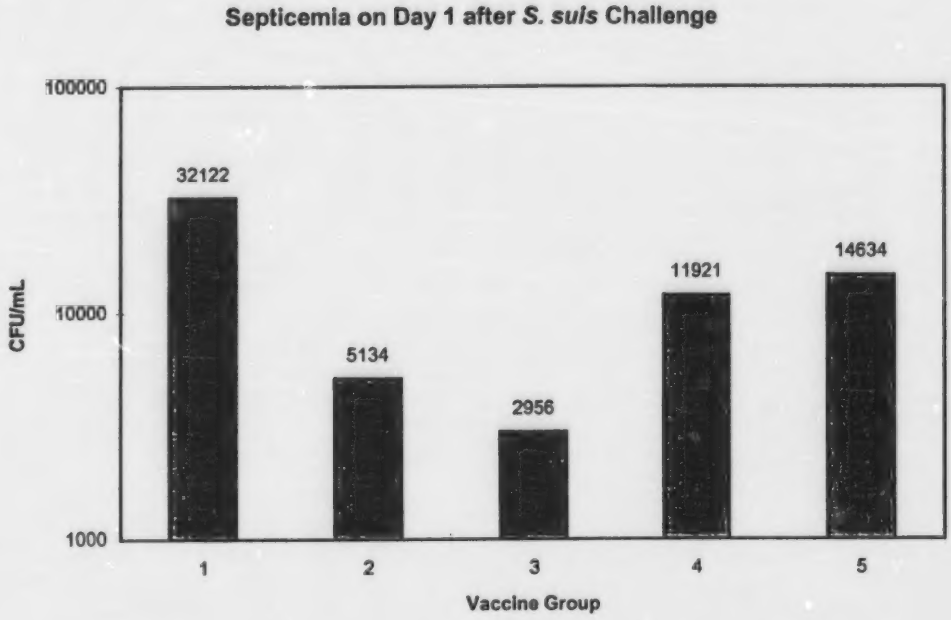
Summary of Characteristics for four *Streptococcus suis* Proteins

Molecular Weight (kDa)	Isoelectric Point (pH)	Pig Id. Number	Specific Antibody Titre	Rabbit Id. Number	Specific Antibody Titre	Protein Identity
44	6.25	57, 58	2500	71, 72	3243	Phospho-glycerate Kinase
56	6.25	59, 60	500	73, 74	4000	
78	6.3	84	500	65	930	
85	5.7	85	500	67, 68	3730	

We have used the protein-specific rabbit sera to show that these serotype 2 proteins are also present in several other strains of *S. Suis*. This work is described in detail in the attached article which was published in the Prairie Swine Centre Annual Research Report for 1996.

c) Initiate vaccine trials using recombinant proteins.

Preliminary screening of the protective effect of these protein antigens requires approximately 1.5 mg of protein. Sufficient protein has been produced using a tac-promoter expression vector to screen the Pyruvate Kinase (truncated), Phenylalanine Synthase, Ornathine Carbamoyl Transferase, Streptococcal Acidic Glycoprotein and 80 kDa-GAP fusion protein antigens. One experiment involved combining the partially purified recombinant proteins with adjuvant, vaccinating pigs at 3 and 6 weeks of age, exposing the vaccinated pigs to *S. suis* bacteria, and monitoring the amount of disease. The pigs mounted a substantial antibody response after the second vaccination to 3 of the 4 experimental vaccines, with a lower level of response to vaccine containing SAGP. Pigs that were given the 80 kDa protein and SAGP vacines (vaccine groups 2 and 3) had less bacteria in their blood (septicemia) after challenge as shown in the graph below. The pigs in vaccine groups 2 and 3 also had and a lower mortality rate than the others.



Hence, the SAGP and 80 kDa protein antigens have shown some protective effect and we continued protein expression work with them. The OCT and Pyruvate kinase proteins do not appear to be protective antigens in this model.

As shown in the table below, we improved protein expression and purification techniques, for a subsequent vaccination and challenge trial to validate the protective effect seen previously

	SAGP Protein		80 kDa Protein	
Purification Stage	Total Protein (mg per Litre)	Estimated Purity (%)	Total Protein (mg per Litre)	Estimated Purity (%)
Crude	80 mg	11 to 17 %	10 mg	< 10 %
Initial Phase	16 mg	25 to 30 %	Unnecessary	Unnecessary
HPLC or Affinity	0.2 mg	> 90 %	2 mg	> 90 %

d) Complete the cloning and expression of *S. suis* genes.

We are working with genes for 4 protective antigens: Streptococcal Acidic Glycoprotein (SAGP), Suilysin, Phosphoglycerate Kinase, and "45" which is the antigenic portion of the "945" fusion protein. Analysis of EC945 clone has shown that it is an inadvertent fusion of two *S. suis* genes. We believe that one of these genes may code for a protective protein since the carboxyl portion of the fusion protein is responsible for reaction with immune serum.

The complete SAGP gene from *S. suis* has been inserted into expression plasmid pGH433 and the complete DNA sequence has been determined. We have identified this gene based upon the similarity of its DNA sequence to that of the SAGP gene from *Streptococcus pyogenes*. This recombinant plasmid results in the production of modest amounts of *S. suis* SAGP protein by *E. coli* bacteria that contain the plasmid. The *S. suis* SAGP protein was used to produce specific immune serum in rabbits (AS#96-034;Rb # 30). The specific immune serum is used to track our progress to increase the production of this SAGP protein.

A portion of the suilysin gene from *S. suis* was amplified from primers that we made using previously published DNA sequence data. This portion of the suilysin gene from *S. suis* has been inserted into pUC18 plasmid and the DNA sequence of this portion has been determined. The inferred amino acid sequence of the portion that we have isolated is slightly different from *S. suis* suilysin that has been previously published. Our sequence is identical for 23 of 23 amino acids at the amino terminal end , but has 6 (of 108) conservatively different amino acids at the carboxy terminal end of the protein. These differences may result in antigenic differences in the suilysin from various strains of *S. suis*. Based on the DNA sequence, the predicted suilysin protein also shows considerable homology to the pneumolysin of *S. pneumoniae*. We have shown by DNA hybridization that this gene is present in 21 of 200 strains of *S. suis* from our collection.

The native Phosphoglycerate Kinase (PGK) protein from *S. suis* has been identified as one component in the protective extract with an apparent molecular weight of 44 kDa. We have produced antiserum in rabbits, which we used to identify several clones in our expression library. Unfortunately, DNA sequencing of several of these clones has indicated that none of them produce PGK.

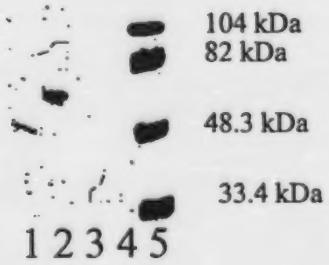
The complete "45" gene from *S. suis* has been inserted into plasmid pGH433 and the complete DNA sequence has been determined. The DNA sequence shows no significant similarity to previously published sequences. A sample of the "45" protein produced by *E. coli* harboring this plasmid was used to produce specific immune serum in rabbits (AS#96-034;Rb # 32). The specific immune serum is used to track our progress to increase the production of this protein. A variety of methods are being used to confirm the identity and function of this protein. The DNA sequence of *S. suis* genes coding for Streptococcal Acidic Glycoprotein (SAGP), Ornithine Carbamyl Transferase and Pyruvate Kinase proteins has been completed.

e) Determine protein expression during infection.

We identified four specific *S. suis* proteins that may be involved in protection from disease as described in our previous report. These proteins were selected for investigation because they are components of an extract vaccine which provided protection from disease. Some of the proteins in the extract vaccine were responsible for the protective effect, but others were not important for protection from disease. We needed to determine which proteins were present and expressed during the infectious process since they are likely to be important candidates for use in a vaccine. Recently, we have confirmed that at least two of these proteins react with the antibodies in serum from an unvaccinated pig which nevertheless recovered from *S. suis* disease.

The Western Blot which is illustrated below shows the reaction of convalescent swine serum with the 44 kDa and 56 kDa *S. suis* protein antigens. The 78 kDa protein was faintly detected but is not visible on the reproduction below. Hence, we will concentrate on identification of the genes for the 44 and 56 kDa proteins.

***Streptococcus suis* Proteins that React with Convalescent Swine Serum**



Lane 1 contains 44 kDa protein which we have identified as phosphoglycerate kinase from *S. suis*. Lanes 2 through 4 contain 56, 78 and 85 kDa proteins respectively. Molecular weight markers of the indicated sizes are in lane 5.

We have completed identification of 6 *S. suis* proteins that are expressed during infection, and they are recognized by reaction with convalescent swine serum. As indicated in the table below, 5 of the 6 identified genes have been cloned and expressed in various amounts. We have produced monospecific rabbit antiserum to each of the 5 proteins and shown that strains of *S. suis* representing 18 serotypes react with each of these sera in western blot reactions.

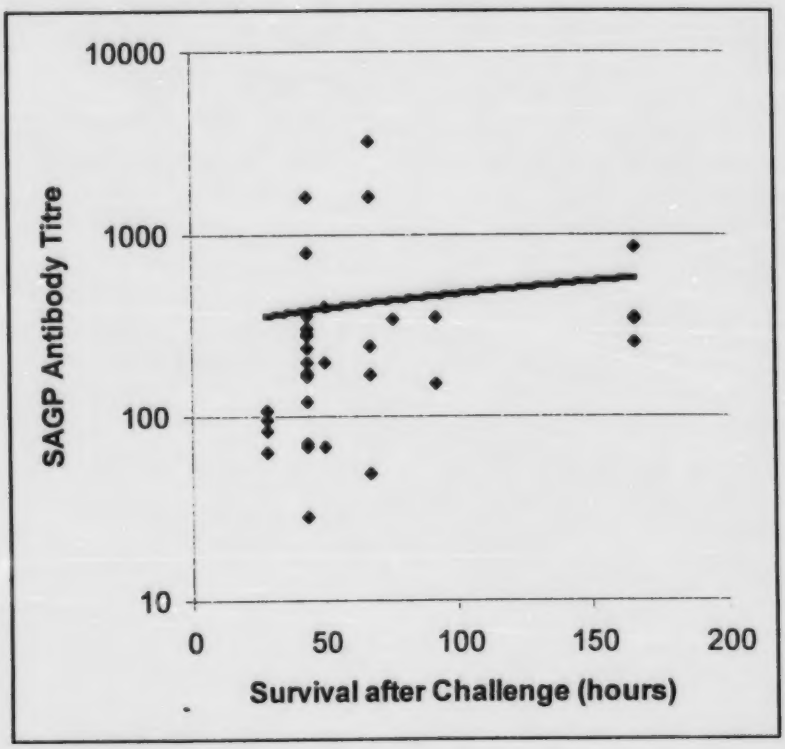
Protein ID	Size (kDa)	DNA Cloning Status
Pyruvate Kinase (truncated)	35	Cloned and expressed
Phosphoglycerate Kinase (PGK)	44	Cloning in Progress
Phenylalanine Synthase	44	Cloned and expressed
Ornathine Carbamoyl Transferase	47	Cloned and expressed
Streptococcal Acidic Glycoprotein (SAGP)	51	Cloned and expressed
1086 Protein (fusion with glutamyl amino peptidase)	80	Expression in Progress

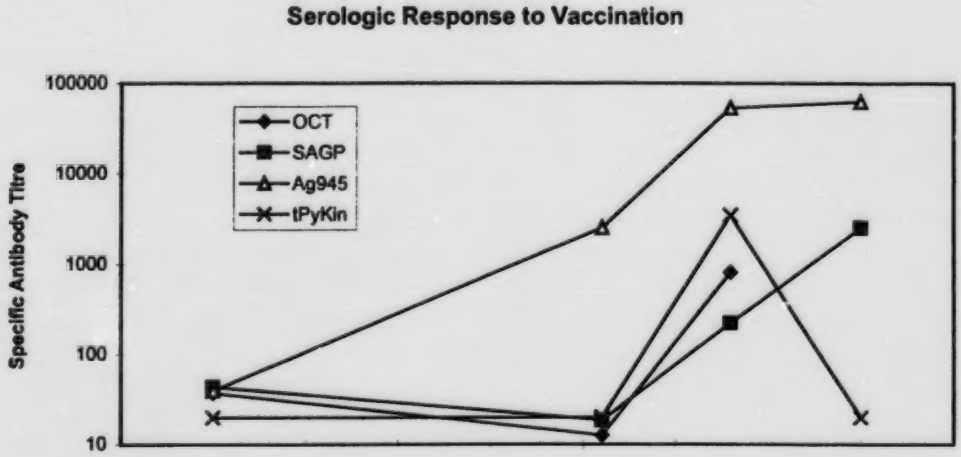
f) Develop ELISA for porcine antibody to specific antigens.

We have based the development of this ELISA to detect swine antibody to recombinant *S. suis* antigens on the ELISA that we used to successfully measure swine antibody to recombinant *Actinobacillus pleuropneumoniae* antigens. We have demonstrated that this test works with four partially purified recombinant *S. suis* antigens: OCT, SAGP, 80 kDa protein and tPyKin. We have

produced higher purity antigens. As expected, these antigens are also fully effective for use in the ELISA. By using more pure antigens, we reduce the contribution of non-specific antibody to the overall antibody titre that is measured. As before, these antigens were used at 1 ug/well to coat 96-well microtiter plates and goat anti-swine alkaline phosphatase-conjugated antibody was used to detect bound swine antibody. The titer was determined to be the reciprocal of the highest dilution that produced an optical density signal greater than 0.025 units above background (no test serum). As shown in the graph below, we have used this ELISA to demonstrate a serological response in pigs that were immunized with partially purified recombinant *S. suis* antigens.

We have measured protection that is provided by immunity to specific proteins work by evaluating the specific relationship between measures of disease severity (amount of bacteria in the blood and duration of survival after challenge) and the level of specific antibody. In this experiment we vaccinated pigs twice (at 3 and 6 weeks of age) with purified recombinant SAGP. The graph below shows that there is a slight positive relationship between increasing specific antibody and duration of survival after challenge with virulent S. suis bacteria.





g) Complete vaccine trials with recombinant proteins using homologous challenge.

Each vaccination and challenge experiment is designed to evaluate protection in pigs vaccinated at 3 and 6 weeks of age with Streptococcus suis vaccine antigens. Each vaccine has protein content of 100 ug/dose in adjuvant. The 2 mL dose of each sterile vaccine is injected intramuscularly (IM). Commercially produced, healthy crossbred piglets without previous vaccination against S. suis are used for this experiment. The piglets are individually identified and randomly assigned to a treatment group (balanced for litter and gender). At weaning each of the piglets is bled, vaccinated and observed for adverse reactions to the vaccination and bleeding procedure. The procedure is repeated 3 weeks later. One week after the second immunization, pigs are challenged by IV injection of 1 mL of *S. suis* (serotype 2 for this experiment). Blood samples are taken to monitor bacteremia or septicemia, and pigs are monitored for clinical signs of disease twice daily for 1 week. All pigs are examined postmortem with histopathology and microbiological culture of selected tissues.

Protein Identification	Gene Cloning Status	Level of Antigen Production	Level of Protection from Disease
PheS	Complete	Ample	None
OCT	Complete	Ample	None
PK	Partial	Ample	None
80-kDa	Complete	Ample	Partial
SAGP	Complete	Limited	Partial
Suilysin	Partial	Not Determined	Not Determined
GAP	Partial	Not Determined	Not Determined
Nuclease	In progress	Not Determined	Not Determined
Fibronectin-binding protein	In progress	Not Determined	Not Determined
PGK	In progress	Not Determined	Not Determined

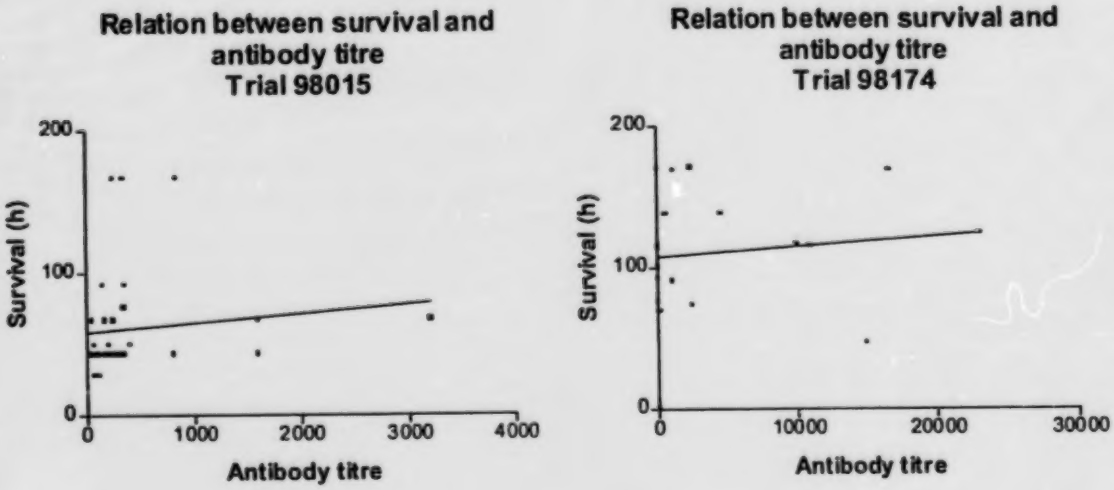
We have conducted a vaccination and challenge trial using 80-kDa and SAGP antigens of at least 75% purity. Although the amount of disease produced in the challenge model was severe, there was partial protection afforded by vaccination with SAGP

Pigs were immunized with a placebo or *S. suis* antigen, and all pigs were anaesthetized and challenged with *S. suis* bacteria by spraying a mist into the back of their throat. Fibronectin in blood is an indication of inflammation that significantly ($p<0.001$) increased after the challenge. These pigs developed disease and two days after the challenge we detected bacteria in the blood of 44% of controls but only 22% of vaccinated pigs. All pigs were evaluated clinically two or more times each day and were humanely killed when they showed signs of disease.

This verified our previous observation of the association between antibody to a purified *S. suis* protein (SAGP) produced using recombinant DNA techniques and survival after challenge.

Unfortunately, this is not a sufficient amount of protection from disease. Nevertheless, we conclude that this antigen may be a useful vaccine component since this was the second trial that has shown a benefit from using vaccine containing this *S. suis* SAGP protein.

The graph below shows that there is a slight positive relationship between increasing specific antibody and duration of survival after challenge with virulent *S. suis* bacteria.



We have completed development of a challenge model of *S. suis* serotype 9 disease in pigs. We anticipate that this model will be used to evaluate whether this vaccine will cross-protect against challenge with another serotype of *S. suis*.

3.1.4 Conclusions:

- We have shown that there is a high prevalence of both infection of pigs and disease caused by *S. suis* in Saskatchewan, Alberta and Manitoba, indicating that immunization with a vaccine that reduced mortality would be an effective and economical method of disease control.

- Despite the high rate of infection with *S. suis* there was not a consistent immune response, highlighting the importance of identifying protective antigens from the bacteria in order to develop an effective vaccine.
- We have shown that vaccination with a special kind of conventional vaccine reduces mortality due to *S. suis* in an endemically infected herd. The approach we used was to vaccinate sows such that antibody against the bacteria would be transferred to piglets through the colostrum.
- The genes coding for several immunogenic proteins contained in this vaccine have been isolated and are currently being characterized.
- Our future work will focus on further characterization of these immunogeinc proteins, including the testing of their protective capacity in swine as well as their ability to protect against the wide variety of *S. suis* serotypes found in the field.

3.1.5 Information of Benefit to Producers:

Streptococcus suis infections in swine are an important cause of loss to producers. During the disease process, or pathogenesis, these bacteria move from the tonsil through the blood stream (septicemia) to the brain and other internal organs and result in death. We have targeted the septicemia phase as a weak link that can be exploited to control this disease. We have demonstrated that pigs with antibody to either of two specific *S. suis* proteins (SAGP and a unique 80-kDa protein) survive longer after experimental challenge with virulent *S. suis*. These results suggest that immunization using a vaccine containing these specific proteins, which will increase specific antibody level, may be a useful tool for producers who need to control *S. suis* infections.

3.2 Project Title: *Escherichia coli* infection in poultry.

3.2.1 Objective:

Our objective is to develop an effective oral vaccine for the prevention of *E. coli* infection in poultry. In the longer term, we will further develop this vaccine as a system for the delivery of antigens from other poultry pathogens in order to produce multivalent vaccines.

Original Timelines:

Year 1: Complete the construction of prototype vaccine strains containing defined mutations.
Year 2: Complete safety testing using prototype vaccine strains.
Determine the protective capacity following oral immunization in septicemia and cellulitis disease models.
Year 3: Complete vaccine testing and experiments designed to determine environmental persistence of the vaccine.
Initiate testing of the vaccine as a delivery system for heterologous antigens.
Year 4: Continue the expression of heterologous antigens including those from Salmonella, *Haemophilus paragallinarum* and mycoplasma.
Year 5: Complete efficacy testing of prototype vectored vaccines.

Revised Timelines:

The original object for year one was completed and the prototype stains tested for attenuation. As neither of the two target mutations lead to adequate attenuation when produced as a defined deletion it was necessary to adopted new target genes. This has resulted in a significant delay in the project and lead to modification of the time lines as listed below.

Year 3: Identify mutation in the *fur* region leading to attenuation
Year 3: Determine efficacy of carbohydrate-utilization mutant as a vaccine for the prevention of disease in broiler and turkeys.
Year 4: Identify nature of carbohydrate-utilization mutant
Construct prototype vaccine strains containing defined mutations.
Safety testing of the prototype vaccines.
Year 5: Determine protective capacity following oral immunization in respiratory colibacillosis and cellulitis disease model.
Initiate testing of the vaccine as a delivery system for heterologous antigens.

3.2.2 Background and Approach:

E. coli infection in poultry is manifested in several forms, including cellulitis and respiratory colibacillosis. This organism is the cause of major economic losses in the poultry industry due to mortality, decreased productivity and condemnation at the time of slaughter. Currently, the only method of disease control is the use of antibiotics. Vaccination would provide an attractive alternative to the use of antibiotics for disease control, and we have shown that oral immunization of poultry with *E. coli* results in protection against disease. Since vaccines for the poultry industry must be produced very inexpensively and be compatible with mass administration, our approach is

to develop a live vaccine in which the bacteria are crippled by the introduction of specific mutations in their DNA.

3.2.3 Results:

The vaccine strains of *E. coli* will contain two independent attenuation mutations to ensure maximum safety. We have investigated four types of mutations including

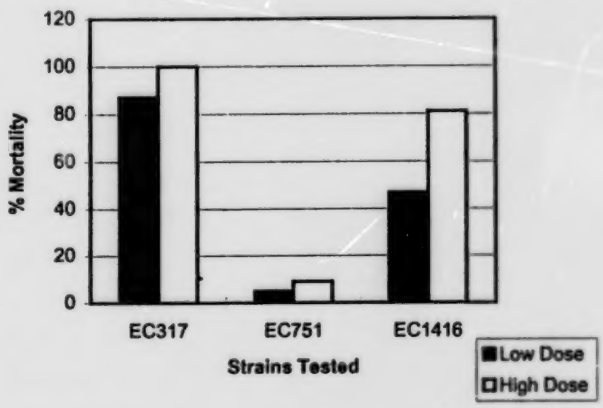
1. mutations of the *car*B gene
2. mutations of the *bar*A gene
3. mutations in the area of the *fur* gene
4. mutations giving rise to altered carbohydrate metabolism.

a) Production and testing of a defined *car*B mutation for attenuation.

Our earlier work had shown that inactivation of the *car*B gene of avian *E. coli* resulted in a decease in virulence as tested in day-old chickens and 7 week-old turkeys. The original mutants had been produced using a transposon mutagenesis technique that made different modifications in each strain (while always inactivating the *car*B gene). As well, some of the modification took place outside the limits of the *car*B gene. These characteristics were considered unacceptable in a strain to be used for vaccine development. To avoid these problems a known deletion of DNA from the *car*B was produced. This known deletion was introduced into the chromosome of a laboratory stain of *E. coli* and transferred to avian strains using generalized transduction.

The resulting strains were then compared to the original wildtype strain of *E. coli* and to the original mutant to determine if they were attenuated (Fig. 1).

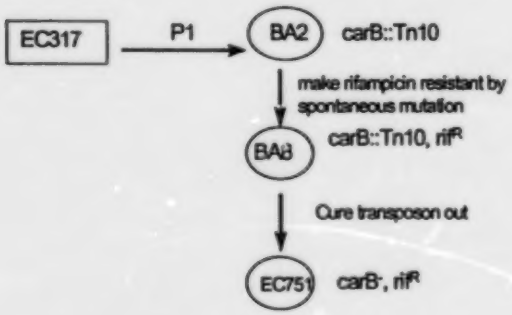
Fig. 1. Virulence of a *car*B Mutant Derived Using a Defined Deletion



It was apparent that the wild type strain (EC317) remained highly virulent and that the original mutant (EC751) was greatly attenuated. The new defined mutants represented by EC1416 were only partially attenuated.

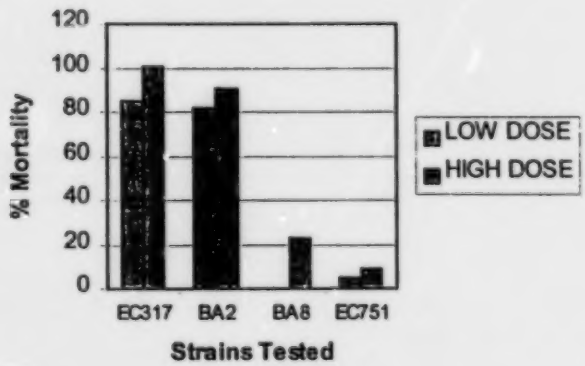
To gain further insight into why the original transposon-derived *car*B mutants differed in virulence from the defined mutants we reexamined the intermediate strains involved in producing the original mutant. (Fig. 2).

Fig. 2. Generation of *carB*⁻ mutants using Tn10



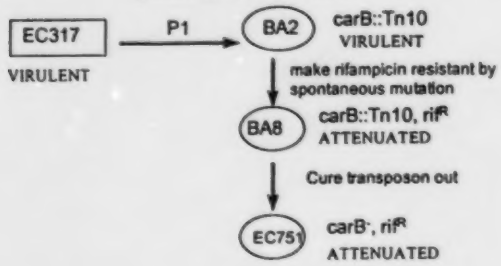
The intermediate strains used in the construction were tested using the young chick model for virulence and were compared to the wildtype parent strain (EC317) and the final transposon derived mutant (EC751).

Fig. 3. Virulence of the strains used to generate the original carB mutants



The intermediate strain that contained only the transposon inserted in the *car*B gene appeared to be fully virulent. In contrast, strains (BA8 and EC751) that were resistant to rifampicin were significantly attenuated. These results were confirmed in mutants derived by this process in two other avian isolates.

Fig. 4. Summary of Virulence *carB*⁻ mutants generated using Tn10



The mutation to produce rifampicin resistance made a significant contribution to the attenuation observed in the original mutants. This contribution to attenuation serves to explain why the second type of *car*B mutants produced were not as attenuated as the original mutants as exemplified by EC751.

Rifampicin resistance is due to a mutation of the beta subunit of the DNA-dependent RNA polymerase. Due to the implications in human health use of a rifampicing resistance mutation in a live vaccine would not be appropriate.

While the defined *car*B mutants were slightly attenuated, we believe that they retain too much virulence for the *car*B mutation to be useful in our vaccine development. We therefore found it necessary to use a different attenuating mutation.

b) Production of a *bar*A mutation in an avian strain.

The BarA protein is a member of the adaptive-response system that contains both the sensory kinase domain and the response regulator domain. This protein is involved in the regulation of the ability of bacteria to adapt to different environments. As such we believe that the inactivation of the *bar*A gene may result in attenuation of the bacteria. From our previous work with the *car*B gene it was clear that to accurately assess the impact of making a deletion within a gene it is necessary to produce a defined mutation. To assess the impact on virulence of the inactivation of the *bar*A gene we attempted to introduce a defined deletion into the gene in a wild-type avian strain. To achieve this aim we obtained a plasmid containing the *bar*A gene. Using this plasmid we removed 899bp of DNA from the gene and cloned the modified gene into a suicide vector. We successfully transferred the defined deletion in the *bar*A gene into the chromosome of a laboratory strain. We have been unsuccessful in transferring the defined mutation into the avian strain. The reason for this is not known. We have discontinued this work to concentrate on the development of other attenuating mutations.

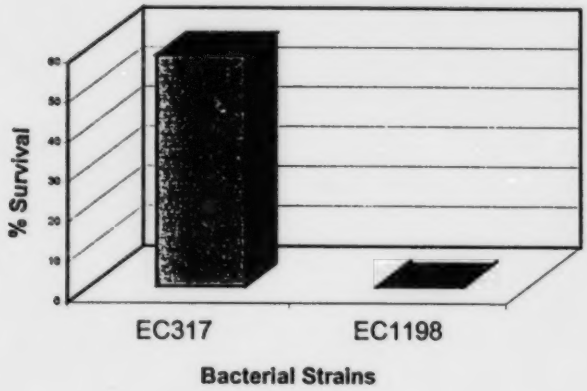
c) Characterization of the nature of the mutation in the area of the *fur* gene.

Preliminary work to examine virulence of *E. coli* containing an uncharacterized *fur* mutation showed the strains were attenuated. When a known deletion was introduced into the *fur* gene and the resulting strains tested no attenuation was observed. In both types of mutants the iron- regulated outer-membrane proteins (IROMP's) were deregulated.

1. Acid tolerance.

A recent report indicated that the role of fur in the acid tolerance response is physiologically and genetically separate from its role in iron acquisition. To determine if our defined deletion mutant was inactivated in both domaines we compared its acid tolerance response to that of the parent strain (Fig. 5).

Fig. 5 Acid Tolerance of the defined fur mutant



It was apparent that the mutant constructed to contain a defined *fur* mutation (EC1198) had become acid sensitive. After exposure to acid conditions for 90 minutes less than 1% of the cells remained viable while almost 60% of the wildtype cells (EC317) remained viable.

2. Sequence analysis.

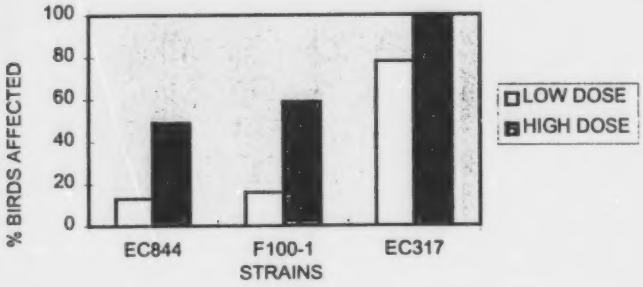
The *fur* gene of the original attenuated strain has been sequenced using DNA fragments amplified by PCR. Primers flanking the entire *fur* gene were used for PCR amplification. Alignment of *fur* gene sequence from three sequencing reactions and *E. coli* K-12 *fur* gene indicated two point mutations in the *fur* gene of the mutant. The mutations result in amino acid substitution (arginine-57 with histidine and glutamic acid-140 with lysine, respectively). No point mutations were found in the promotor region of the *fur* gene. These minor alterations are consistent with what would be expected in a mutation produced by NTG mutagenesis.

3. Complementation studies.

We have previously determined that the inactivation of the *fur* gene using a defined deletion did not lead to attenuation of avian strains. In contrast, the *fur* mutants produced by chemical mutagenesis were attenuating. Since the chemically produced *fur* mutants were transferred into the avian strain using generalized transduction we know that the alterations which produced the observed attenuation are adjacent to the *fur* gene. To determine the changes in this region that lead to the attenuation originally observed we carried out a complementation study using F' factors. Three F' factors that cover the region of interest were tested for their ability to restore virulence to the avian strain and compared to the

virulent wildtype strain (EC317) and the attenuated mutant (EC844). Initial experiments suggested that the presence of F'100-1 significantly increased the virulence of the mutant. Subsequent experimentation has failed to reproduce these results. In some experiments a small difference was observed but it was not statistically significant.

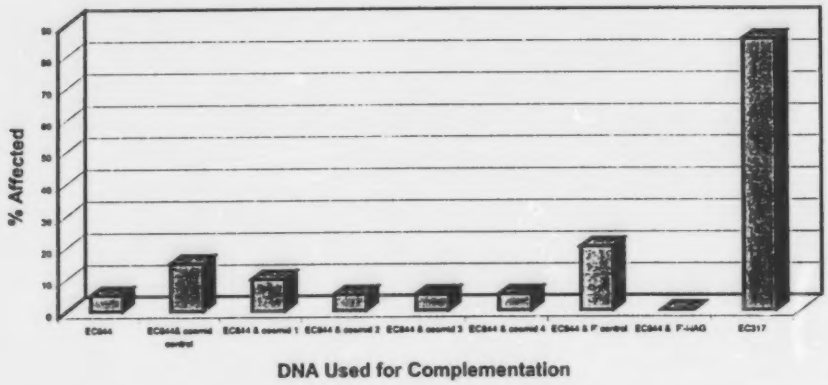
Figure 6. Complementation of Virulence of EC844 using the F' Factor F100-1.



It appears that complementation using F' factors derived from this region of laboratory strains do not restore virulence to the mutant (EC844).

Further analysis was undertaken using F' factors derived from the avian parent strain EC317 to ensure an important virulence factor is not missing in the laboratory strains. In addition, complementation using smaller pieces of DNA (cosmids) derived from EC317 is being carried out.

Figure 7. Complementation Analysis of Virulence of EC844 using an F' Factor and Cosmids derived from EC317.



EC844 is the original attenuated mutant derived from the wildtype strain EC317. The cosmid control was a cosmid from an unrelated region of the chromosome of EC317. Cosmids 1 and 2 contained DNA from the *nag* gene area of EC317. Cosmids 3 and 4 contained DNA from the *nag* gene and the *sdh* gene of EC 317. These genes flank the *fur* gene. The cosmid F'-NAG contains DNA that includes the *nag* gene of EC317. None of these restored virulence to the mutant strain EC844.

The approach of complementation to define the mutation in the area of the *fur* gene has not been successful. To further explore the area of this mutation we are currently cloning the area from the original mutant strain and will undertake sequence analysis of the region.

4. Localization of the Tn10 insertion.

The original *fur* mutation had been marked by the insertion of the transposon Tn10 in an area adjacent to the *fur* gene. The exact location of the insertion had not been determined. The Tn10 insertion may contribute to the loss of virulence originally observed. Using reverse PCR and sequencing we have determined that the Tn10 is inserted into the *kdpE* gene. We are currently producing a defined mutation in this gene so that we can test the effect of inactivation of the *kdp*E gene on virulence.

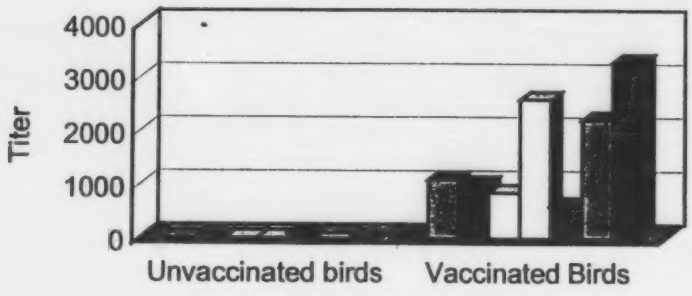
d) Development of an ELISA system to detect humoral response in poultry.

We have developed an enzyme-linked immunosorbent assay (ELISA) test for serotype-specific antibodies to *E. coli* that utilizes lipopolysaccaride (LPS) as the antigen. LPS is the heat stable antigen that the O serogroup system for *E. coli* is based on. The initial development was done using antisera that were produced in rabbits and were of high titer. Using this antisera we optimized the concentration of various components of the system and determined that crossreactivity occurred only at a very low level. Using this LPS-ELISA system we have detected antibody to a particular serotype of *E. coli* even in a sample of broiler serum that contained antibodies to other serotypes of *E. coli.* This assay will be used to determine if there is a correlation between protection observed in our animal models after vaccination and level of antibody to challenge strain that is present An example of the use of this test is shown below.

Turkeys were orally vaccinated with live *E. coli* at 4 weeks of age. Sera was collected prior to vaccination, at 7 weeks of age just prior to challenge and one week after challenge. Post vaccination sera collected from four turkey challenge experiments was analyzed using the ELISA system developed at VIDO to determine if oral vaccination resulted in an increase in *E. coli* antibodies present.

Considerable variation was observed in titers in both the vaccinated and unvaccinated birds. The vaccinated birds as a group appeared to have higher titers than the unvaccinated controls.

Fig. 8.Titer of Turkey Sera Before Challenge

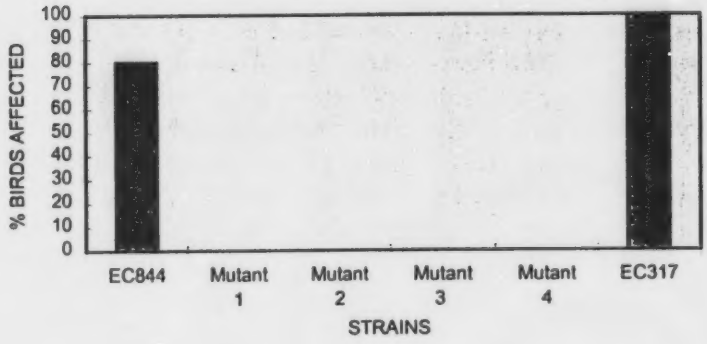


e) Production and testing of a avian *E. coli* containing a carbohydrate utilization mutation.

1. Testing of the effect of alteration in carbohydrate utilization on the virulence of avian *E. coli*.

Previous work by other researchers has shown that this class of mutations is effective in reducing virulence in other bacteria including *Salmonella enteriditis*. We produced several mutants of EC844 that had altered carbohydrate utilization profiles. After testing for stability of the mutation, four of these mutants were tested in the one-day old chick model for changes in virulence.

Figure 9. Effect of Changes in Carbohydrate Utilization on Virulence.



The carbohydrate utilization mutants appeared to be greatly reduced in virulence. No animals tested died when challenged with these strains. In contrast, the parent strain (EC844) affected 80% of the birds and the highly virulent wildtype strain (EC317) affected 100% of the birds.

The efficacy of strains with altered carbohydrate metabolism to function as a vaccine for the prevention of disease caused by *E. coli* in broilers was tested.

2. Testing of the efficacy of mutant *E. coli* with altered carbohydrate metabolism as a vaccine.

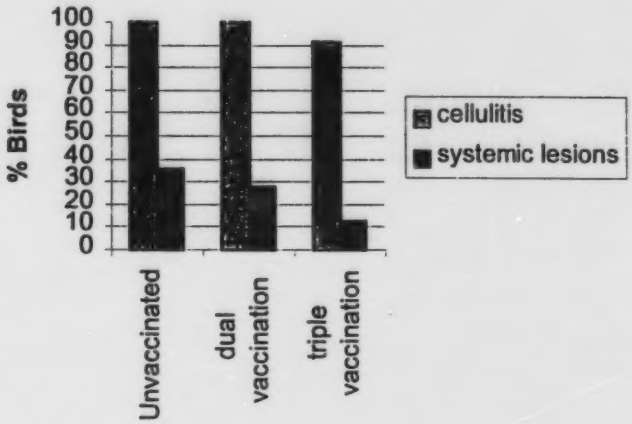
Three vaccination strategies were tested as outlined below.

Group	Aerosol vaccination Day 1	Oral vaccination Day 14	Oral vaccination Day 28
Control	NO	NO	NO
Oral vaccination	NO	YES	YES
Oral and aerosol vaccination	YES	YES	YES

All birds were challenged on Day 35 by the subcutaneous injection of *E. coli* in the lower abdomen. Birds were monitored for systemic disease and those that developed the

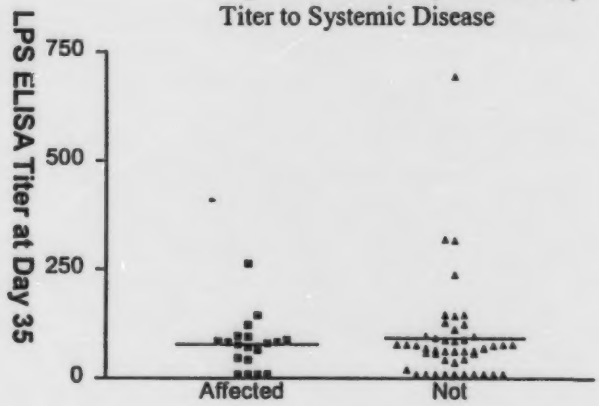
predetermined clinical signs were euthanized. All remaining birds were euthanized and examined for lesions at day 7-post challenge. Vaccination had no effect on the occurrence of cellulitis in the birds. As well dual vaccination had no effect on the occurrence of systemic lesions. The triple vaccination strategy appeared to cause a small reduction in number of systemic lesions (p= 0.07)

Figure 10. Occurrence of Lesions After Challenge with *E. coli.*



The antibody titers were determined using an ELISA technique with LPS isolated from the same bacteria that was used for vaccination. The titers were sorted according to outcome of the challenge (systemic disease or no systemic disease) after challenge. The titer in the sera of birds just before challenge was not predictive of the outcome of the challenge (Fig.11).

Fig. 11. Relationship of Antibody Titer to Systemic Disease



The horizontal line for each group indicates the mean.

f) Characterization of the virulent avian *E. coli* that will be used as the basis of the prototype vaccine.

E. coli EC317, a highly virulent isolate from respiratory colibacillosis will be used as the basis of our prototype vaccine. The appropriate mutations will be inserted into this strain. It is important, due to licencing considerations, to use a well characterized strain of *E. coli* in the prototype vaccine. We have determined that this strain of *E. coli* contains three plasmids. The large (approximately 70kb) plasmid appears to be related to the ColV plasmid. It contains the *tsh* gene and confirs both the ability to grow under low iron conditions and serum resistance to the host strain. There are also two small plasmids of 4kb and 2kb. The two small plasmids have been sequenced. The smaller of these two plasmids appears to be a small cryptic plasmid (pKL1) previously described in the literature while the 4 kb plasmid has significant homology to the expression vector pWLG14.

g) Identification and characterization of a new type of cellulitis in turkeys.

Inspectors have observed a new form of cellulitis lesions in turkeys. This new form of lesion closely resembles cellulitis observed in broiler chickens. To date little information has been collected on the causative agent(s) of these lesions. At this time cellulitis in turkeys is a relatively minor problem but based on the experience with broilers where cellulitis has increased dramatically it should not be ignored.

Fourteen flocks from eight producers were sampled at the local processing plant and 25 turkeys with cellulitis on legs or caudal thoracic area were obtained. This sampling was done in conjunction with Dr. L. Belanger and Dr. B. Althouse from the Canadian Food Inspection Agency. On gross examination none of the birds with cellulitis had lesions in other organs. After gross examination the cellulitis lesions were divided into two groups:

1. cellulitis with open skin lesions
2. cellulitis with unopened skin lesions

The lesions were sampled for aerobic and anaerobic bacteria using standard techniques. All samples were inoculated onto growth media within 4 hours of being taken. Bacteria were isolated from 12 of the 25 birds with cellulitis lesions. No aerobic, microaerophilic or anaerobic bacteria were isolated from the remaining birds with cellulitis lesions.

No aerobic and anaerobic bacteria were isolated from the pericardium, liver or air sacs.

h) Identify and Characterize DNA that is unique to *E. coli* isolates that are pathogenic for poultry.

As reported previously, there are important, but so far uncharacterized, differences between laboratory strains of *E. coli* and strains of *E. coli* that cause disease in poultry. This aspect of our research investigated the characteristics and function of the DNA that is unique to *E. coli* that cause disease in poultry. We will also determine how widespread these unique sequences are in other groups of *E. coli* that cause different types of disease in poultry.

A well-characterized laboratory strain of *E. coli* and two isolates that are known to be pathogenic for poultry were employed in subtractive hybridization techniques using the Clonetech PCR-select bacterial genome subtraction kit.

The PCR products were cloned into the pT-Adv plasmid (Clonetech), which is designed specifically for cloning PCR products. Plasmid DNA was isolated from 1000 clones of which 210 were shown to contain inserted DNA. The inserts vary in size from 300 to greater than 2000 base-pairs of DNA.

Southern blot analysis confirmed that 67 of the inserts were unique to *E. coli* strains isolated from poultry. These sequences were further characterized into one of 5 types as shown in the table below.

Subtractive hybridization sequence summary

Type of Sequence	Homology	Number of Inserts
Phage	various	26
Plasmid	various	3
	E. coli/ColV-K30 plasmid	2
	E. coli plasmid EAF	1
Transposons	various	5
Known Sequences	*B. subtilis* probable fructose-bisphosphate aldolase	1
	S. dysenteriae shuV/U/Y/X/W/A/S genes	1
	E. coli genes for F13 P-pili proteins	1
	E. coli finD gene type 1 fimbrae	1
	E. coli UDP-glucose LPS	1
	E. coli csvR gene	1
	E. coli hemolysin D (hlyD) gene	1
Unknown Sequences	none	22

Collaborative Research:

During this time we have collaborated with a number of other groups to facilitate their research for the poultry industry. In most cases we have provided bacteriology expertise to pathologists or nutritionists.

- We have provided bacteriology support to Dr. C. Riddell and Dr. B. Goodhope WCVM during their work on cellulitis in chickens.
- We have also consulted with and provided material to Dr. D. Krover, U. of Alberta, for his investigations of cellulitis in broilers.
- We were actively involved in a study on cellulitis done in Alberta by Dr. Onderka and Dr. Hanson from Alberta Agriculture and with Dr. K. McMillan (Lilydale Poultry).

Publications, Presentations and Patents:

Eight papers have been published based on the information produced by this research and we anticipate that two more will be published We have also made 24 presentations including invited talks to the Alberta Poultry Service Industry Workshop, The Atlantic Poultry Conference and the Saskatchewan Poultry Industry Conference. The remainder of the presentations were made ate scientific meetings in Canada and the United States. Two patents (United States Patent No. 5,747,309 and European Patent No. 0659 086) have been issued.

3.2.4 Conclusions:

1. Oral vaccination of turkeys has been shown to be effective in the prevention of respiratory colibacillosis in turkeys.
2. Vaccination of broiler under experimental conditions was not effective in reducing the occurrence of cellulitis.
3. Introduction of a defined mutation in the *carB* or *fur* gene does not result in significant reduction of virulence in the avian *E. coli.*
4. The cellulites lesions observed in turkeys do not appear to be due to a bacterial infection and are therefore different from those observed in broilers.
5. *E. coli* capable of causing disease in poultry contain many sequences that are not found in a non pathogenic strain. The nature of these unique sequences and their contribution to virulence require further investigation.

3.2.5 Information of Benefit to Producers:

<u>*E. coli* vaccine development:</u>

- Developed a model for infection of turkeys that mimics respiratory colibacillosis observed in the field. Approximately 75% of the animals develop disease. With lesions that are the same as seen in natural disease. Turkeys are predisposed to disease by exposure to hemorrhagic enteritis virus which is like what happens in the field.
- Characterized *E. coli* isolated from birds in western Canada with colibacillosis and found that they had the same characteristics as those from other parts of the world.
- Showed that oral vaccination is effective in preventing systemic disease. This was done using an experimental vaccine containing virulent *E. coli* that is unsuitable for use in the field.
- Developed an ELISA test to detect *E. coli* antibodies. This is useful in following the response to vaccination both in chickens and in turkeys.
- We have developed two models for the experimental reproduction of cellulitis in chickens. These models have been used by other researchers in Alberta for their work. as well as in our own studies.
- We have been unable to show any impact (under experimental conditions) on the occurrence of cellulitis using vaccination. We have also observed that there was no correlation between the level of antibodies produced and protection.
  During the course of these studies we also observed no impact of vaccination on the occurrence of systemic disease. Thus, we believe that management practices will be a more effective control method than vaccination.

<u>Cellulitis in turkeys:</u>

A new type of lesion in turkeys that appeared to have similar characteristics as those in broilers was described around 1998. These lesions occur at a very low level at this time but due to the concern that they would increase in frequency (as happened in the broiler industry) we extended the original report to sample lesions to determine if *E. coli* was also involved in the occurrence of the lesions. It appears that the lesions are not due to an infection but to a foreign body contamination of the skin.

Identification of genes that are unique to *E. coli* pathogenic for poultry:

This work is part of the graduate project of S. Stocki. It is anticipated that she will graduate in December 2001. She has identified many small pieces of DNA that are not found in a laboratory strain of *E. coli* but that are found in the strains that are pathogenic for poultry. Some of these unique pieces of DNA may contribute to the virulence of the bacteria. The nature of selected unique sequences is being investigated.

3.3 Project Title: Control of bovine mastitis.

3.3.1 Objective:

Our objective is to develop a comprehensive vaccine for bovine mastitis due to contagious and environmental bacteria. The short-term objectives are to develop subunit vaccines for the environmental streptococcal species, *Streptococcus uberis,* and *Streptococcus dysgalactiae.*

3.3.2 Background and Approach:

Bovine mastitis is an inflammation of the mammary gland and is the leading cause of economic losses to the Canadian dairy industry. These losses have been estimated at >100 million annually due to the loss of production and reduction in milk quality. For well-managed herds in which the rate of contagious mastitis is low, infection with the so-called environmental streptococci is responsible for up to 25% of clinical cases. Vaccination is likely to be the only effective method of disease control for these organisms because of their presence in the environment and also due to pressures to reduce antibiotic use in food-producing animals.

Our approach to vaccine development is to define specific factors produced b the bacteria involved in disease and to produce sufficient quantities for vaccine testing using recombinant DNA technology. Once individual antigens have been identified, we will combine them to produce a combination Streptococcal vaccine. Once is complete, we anticipate using a similar approach to produce subunit vaccines for *E. coli* and/or *Staphylococcus aureus* mastitis.

Timelines:

Year 1:
- Identification of *Streptococcus uberis* and *S. dysgalactiae* antigens.
- Vaccine testing of antigens from both organisms.

Year 2:
- Cloning and expression of genes coding for protective antigens from both species.
- Vaccine testing of antigens from both organisms using heterologous challenges.

Year 3:
- Identification of *Streptococcus agalactiae* antigens.
- Vaccine testing using heterologous challenge models.
- Test combination *S. uberis-S. dysgalactiae* vaccines for protection in homologous and heterologous challenge models.
- Determine the optimal vaccine formulation and method of delivery.

Year 4:
- Cloning of genes coding for *S. agalactiae* antigens.
- Identification of *Staphylococcus aureus* antigens.

Year 5:
- Continue with the cloning and expression of genes coding for *S. agalactiae* and *S. aureus* antigens.

3.3.3 Results:

a) Identification of *Streptococcus uberis* antigens.

We have identified four antigenic *S. uberis* proteins, lactoferrin-binding protein (Lbp), CAMP factor, R Antigen, and GapC. Similar to other pathogenic bacteria, *S. uberis* requires iron for growth. Cattle restrict the availability of iron by coupling it to various proteins such as lactoferrin that is present at high concentrations in milk. We have characterized the interaction

between *S. uberis* and bovine lactoferrin, and determined that there are approximately 7800 binding sites on each bacterial cell. These proteins may act as receptors for nutritional iron or to signal metabolic changes in the bacteria which enable it to cause mastitis as it moves from the environment into the gland. In either case, Lbp is a good vaccine candidate. An expression library was produced and a recombinant clone that binds to bovine lactoferrin has been identified.

The CAMP factor was originally identified from Group B Streptococci in 1944. It is present in strains of *S. agalactiae* and *S. uberis* that cause mastitis. The CAMP factor protein was purified from *S. uberis* and used to produce specific antiserum. Specific serum cross-reacted with *S. agalactiae* protein B and demonstrated the CAMP factor in 3 or 8 strains of *S. uberis*. This gene has been cloned and sequenced in our lab.

The R antigen of *S. uberis* shares some similarity to the M-proteins that are virulence attributes of Group A Streptococci. The R Antigen binds non-specifically to immunoglobulin (this characteristic is shared by other Fc-binding proteins) and lacks another identifiable characteristic. We purified R antigen of *S. uberis* for production of specific immune serum with the purpose of obtaining monoclonal antibodies that could be used in the cloning of the R antigen gene but we did not succeed at this task. Thus we abandoned the attempts to clone the gene for the R Antigen at this time since specific reagents could not be produced.

The amino acid-binding protein (Abp) is a protein located in the periplasmic space of the bacterial cell and involved in the transport of amino acids to the interior of the cell.

The GapC protein has been identified as a surface-exposed antigen that could bind bovine plasmin and other mammalian proteins. In addition, the GapC homologue in Group A streptococci has been postulated to play a role in virulence of these bacteria and new evidence suggests that antibodies against the GapC homologue in *Schistosoma mansoni* confer protection against a reinfection by this parasite. These observations prompted us to consider the *S. uberis* GapC protein as a vaccine candidate against mastitis.

These results complete the objective of identifying and isolating antigens from *S. uberis*.

b) Identification of *Streptococcus dysgalactiae* antigens.

S. dysgalactiae has specific receptors for bovine vitronectin, an interstitial protein. The binding to vitronectin may be an important step in colonization of the bovine gland. We have used labelled detection methods to show specific interaction between the *S. dysgalactiae* cell and vitronectin and we planned to use the same detection method to identify the *S. dysgalactiae* gene that encodes the receptor but our efforts proved to be unsuccessful. The gene coding for the vitronectin-receptor will not be isolated until specific reagents for the detection of this protein are available. We have cloned *S. dysgalactiae* genes for 5 proteins: Fibronectin-binding protein A and B, Mig, Mag, and GapC. The fibronectin-binding proteins are surface-expressed products presumably involved in the virulence of the bacteria by binding the serum-protein fibronectin and helping the bacteria to evade the immunological surveillance of the host.

The Mig protein is also surface-expressed and it belongs to a group of proteins that bind IgG by their Fc receptors. Mig can also bind the plasmin-regulator alpha-2-macroglobulin. We have found that in addition to its binding properties, Mig plays a role in virulence by conferring resistance to phagocytosis and intracellular killing by PMNs. Because of these properties Mig could be a potential target for vaccine development.

The Mag protein is also surface expressed and like Mig binds IgG by the Fc receptors. The other serum protein that binds to Mag is albumin and the binding of these two serum products to the cell surface may help the bacterium to evade the immune response of the host.

The GapC protein has also been identified in *S. dysgalactiae* and although it shares a high degree of homology with the GapC of *S. uberis*, there are parts that are not identical between these two proteins. Because of the GapC properties listed above, this protein is also a good candidate for developing a vaccine against mastitis caused by *S. dysgalactiae*.

These results complete the objective of identifying and isolating antigens from *S. dysgalactiae*.

c) Identification of *Streptococcus agalactiae* antigens.

We have also isolated the gene encoding the CAMP factor in *S. agalactiae*. This protein shares an extensive area of homology with the CAMP product of *S. uberis* but there are regions that are not conserved.

The gene encoding for the GapC protein of *S. agalactiae* has also been cloned. The GapC protein is highly identical to the GapC products of *S. uberis* and *S. dysgalactiae*. However there are regions of the protein that are unique. Because of the GapC properties listed above, this protein is also a good candidate for developing a vaccine against mastitis caused by *S. agalactiae*.

These results complete the objective of identifying and isolating antigens from *S. agalactiae*.

d) Construction of chimera antigens.

Despite that the GapC proteins of *S. uberis*, *S. agalactiae*, and *S. dysgalactiae* share considerable homology, there are amino acid sequences that are unique to each protein. In order to ensure that a GapC-based vaccine can be used to protect against both the environmental and well as the infectious streptococci and to make certain that vaccine production can be carried out in a economically feasible fashion, we have constructed chimeric genes composed of portions of the three *gapC* genes. One of the chimeric proteins uses the *S. dysgalactiae* GapC, and the other the *S. uberis* GapC as backbones.

The CAMP proteins of *S. uberis* and *S. agalactiae* share extensive sequence homology except for a region in the middle of the proteins that is unique to each protein molecule. A chimera protein has been constructed with the CAMP product of *S. uberis* as the backbone to which the non-identical region of the *S. agalactiae* CAMP has been added. Both, the GapC and CAMP chimeras are being expressed in *E. coli* by a unique mechanism that will be compatible with adding *E. coli* vaccine components to this formulation at a later date.

e) Initiate vaccine testing of antigens from *S. dysgalactiae* and *S. uberis*.

We have tested CAMP and Lbp recombinant protein antigens as vaccines to protect dairy cows from *S. uberis* mastitis. Only one of these recombinant antigens (CAMP) induced an antibody response in vaccinated cows. Furthermore, those cows vaccinated with CAMP were resistant to inflammation of the mammary gland (mastitis) after challenge with *S. uberis*. Although the distribution of CAMP-factor among strains of *S. uberis* is limited, antibody to this antigen may also neutralize CAMP-factor from another mastitis pathogen, *S. agalactiae*. The second antigen (lactoferrin-binding protein) did not confer an acceptable level of protection in a heterologous

disease model. Thus, we required the addition of a second *S. uberis* antigen that it could be combined with the CAMP factor.

We carried out a dose titration trial to determine the optimum amount of the CAMP antigen to use for the vaccine. As shown in the graph below, each group of vaccinated cows had an antibody response. The average titre in the cows that were given the 4 ug dose was not as high or as persistent as the antibody level in the groups given a larger dose. Hence we have concluded that an optimal dose is between 20 and 100 micrograms.

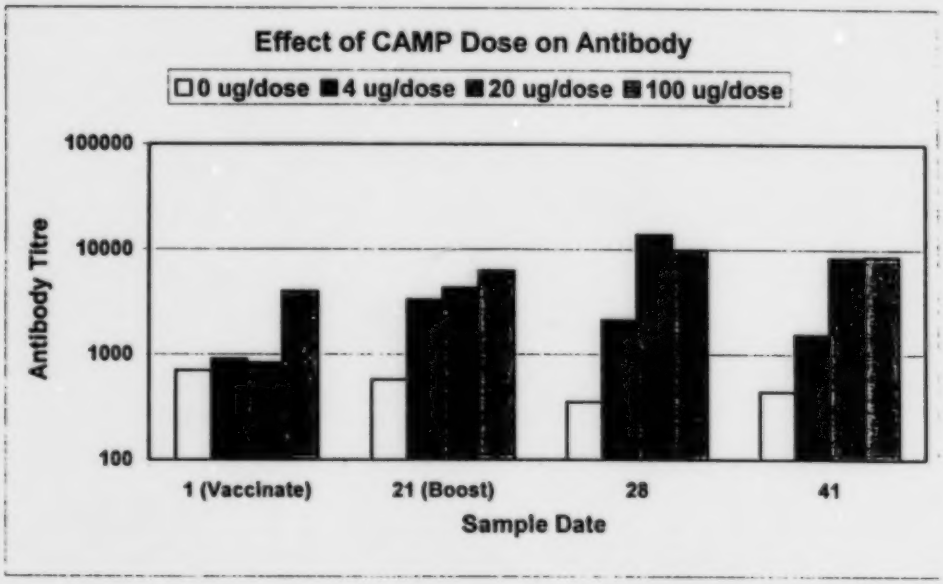


Figure 1. Antibody response to CAMP. Serum antibody titres at days 1, 21, 28 and 41 after immunization with increasing amount of CAMP protein.

One of the difficulties with development of a vaccine to prevent *S. dysgalactiae* mastitis is that the disease only occurs in cows in the early dry period. We first performed a preliminary *S. dysgalactiae* vaccine trial with antigen that consisted of *S. dysgalactiae* cell walls. The level of protection was unclear from this trial and thus we decided to repeat it using purified antigens.

During late 1998 we conducted a vaccine trial to test the protective capacity of the *S. dysgalactiae* FnbB, Mig and GapC antigens in an experimental challenge model. The high level expression of GapC protein has enabled us to complete this trial for evaluation of experimental *S. dysgalactiae* antigens produced using recombinant DNA technology. As shown in the graph below, there was a strong relationship between high levels of antibody and protection from infection. That is, cows with high antibody levels had fewer infected quarters after challenge.

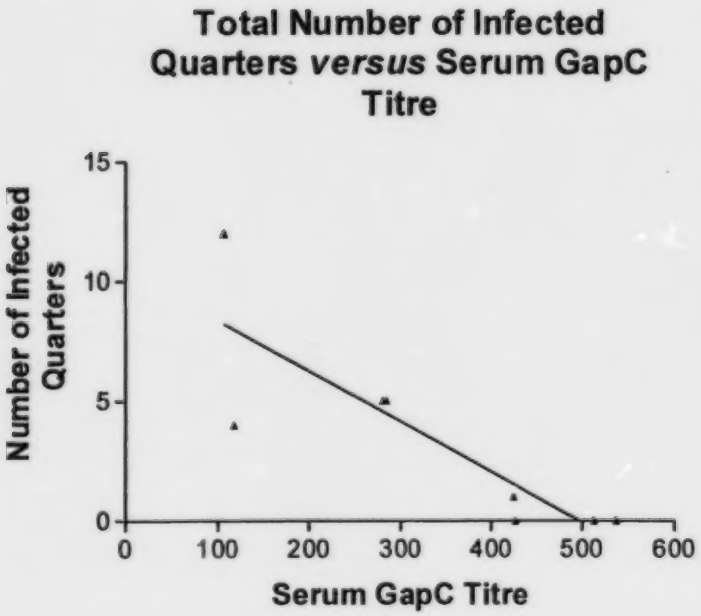


Figure 2. Effect of serum titres against GapC on protection against mastitis. The serum titre against GapC was compared to the number of infected quarters in the same animal.

f) Vaccine testing using heterologous challenge models.

In late 1998, we have completed a vaccine trial to test the ability of the *S. uberis* CAMP Factor and Lbp to protect against challenge with a different *S. uberis* strain from that which was used for gene cloning. This is important in order to demonstrate the effect that vaccination is likely to have against disease caused by the wide variety of strains that occur naturally. The trial was conducted using the following design:

Group A: 2 cows given placebo
Group B: 3 cows doubly vaccinated with CAMP in Emulsigen-Plus
Group C: 3 cows doubly vaccinated with Lbp in Emulsigen-Plus
Challenge with heterologous strain of *S. uberis* (SU-21)
Provide therapy as needed and monitor for 7 days

Antibody Response: Each principal group had significant antigen specific response (that is to either CAMP or Lbp) and no significant response to Whole Cell Antigen.

Body Temperature: All groups were similar with no occurrence of fever and slight (but significant) decrease during the trial; apparently they got used to the procedure and became less nervous. There was no indication of systemic disease and no effect of group.

Milk Production: Daily milk production (kg/day) was (significantly) less in Group B both before and after challenge. There was no effect of time (and no effect of challenge). Allocation of individual cows to vaccine group was done prior to measurement of daily milk production, and evidently the cows that were allocated to group B produced less milk from the outset.

Measurements of mastitis: California Mastitis Test and Somatic Cell Counts on the same sample were well correlated and highly significant. Control group had significantly higher CMT score (over all times). There is a significant effect of time on SCC (highest at 48 and 72 hours after challenge) but no significant effect of group.

Isolation of SU-21 and mastitis: There was a small (3% of the effect), but significant correlation between isolation of SU-21 and SCC. No significant association between challenge (or not) and SU-21 isolation (or not). There was no significant correlation with total bacteria and mastitis (either CMT or SCC). The control group had significantly more of the bacteria given for challenge on 2 samples (24 & 48 hours after challenge).

The greatest impact was on treatment for mastitis: Group B (CAMP Vaccine) had significantly lower proportion of quarters treated for mastitis during the vaccine trial as shown in the graph below.

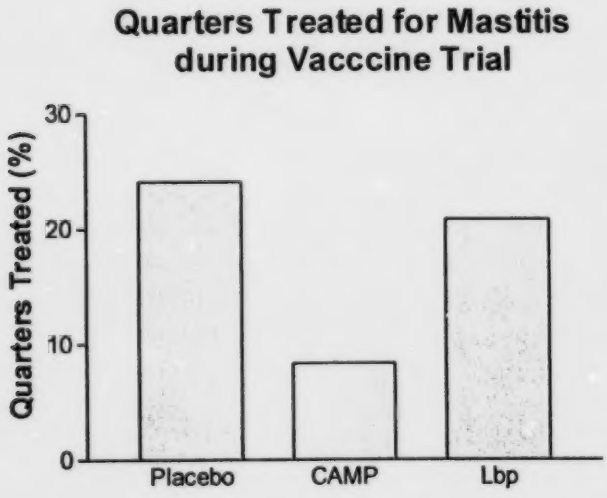


Figure 3. Percentage of quarters treated for mastitis. Animals were immunized with CAMP, Lbp and a placebo and challenged with *S. uberis* Su21.

We have previously demonstrated the protective capacity of the *S. uberis* CAMP factor antigen in homologous and heterologous disease models. However, a second antigen (lactoferrin-binding protein) did not confer an acceptable level of protection in a heterologous disease model. Thus, we required the addition of a second *S. uberis* antigen that could be combined with the CAMP factor. For *S. dysgalactiae*, we have completed the identification of specific vaccine targets and cloning and expression of the appropriate genes. During the vaccine trial conducted during 1999

we demonstrated that the GapC and Mig proteins were able to confer protection against experimental infection with *S. dysgalactiae*. These results are illustrated below.

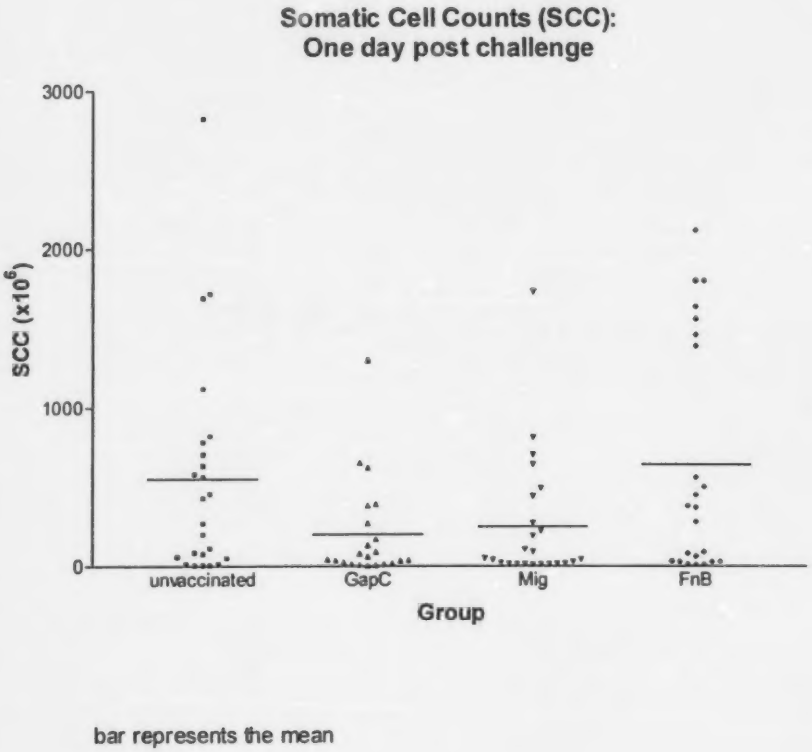


Figure 4. Protective capacity of *S. dysgalactiae* antigens. Somatic cell counts per quarter on one day post-challenge. Animals were immunized with GapC, Mig or FnB and then challenged by intramammary infusion of *S. dysgalactiae*.

We had previously described the construction of chimeric genes containing portions of the GapC protein of *S. uberis*, *S. dysgalactiae* and *S. agalactiae* as well as a chimeric gene expressing CAMP factors of *S. uberis* and *S. agalactiae*. These genes have been constructed in order to obtain broad-spectrum protection against mastitis-causing organisms using a minimal number of antigens in the vaccine. We have demonstrated proof of concept for immunization with the *S. uberis* CAMP factor as well as the *S. dysgalactiae* GapC and Mig proteins. During the past year, we have focused on testing of the *S. uberis* and *S. agalactiae* GapC proteins as potential vaccine components as well as the chimeric CAMP factor molecule. Three large vaccine trials were initiated during the summer of 2000, designed to confirm the ability of GapC and Mig to protect against *S. dysgalactiae* infection and the ability of GapC and the CAMP factor to protect against both *S. uberis* and *S. agalactiae* infection.

The *S. dysgalactiae* vaccine trial involved immunization of animals with the *S. dysgalactiae* GapC protein, the *S. uberis* GapC protein, Mig, *S. dysgalactiae* GapC plus Mig or a placebo. The

SCC in all the un-challenged quarters were compared to the SCC obtained from the challenged quarters of each individual group. The results indicated that immunization with any of the vaccines resulted in a beneficial effect following experimental challenge with a heterologous strain of *S. dysgalactiae* as shown in the figure below.

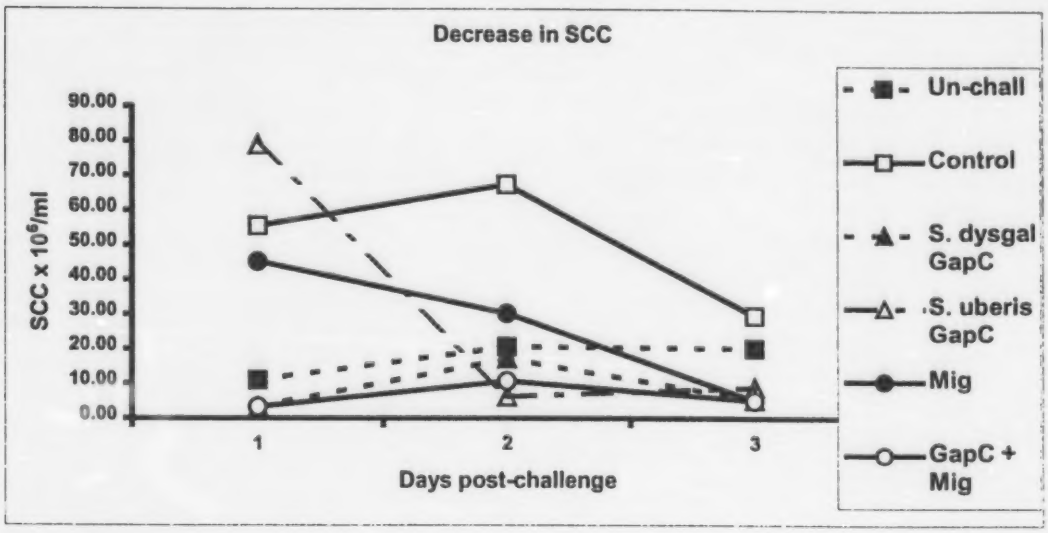


Figure 5. Protective capacity of *S. dysgalactiae* antigens. Somatic cell counts per quarter on three days post-challenge. Animals were immunized with *S. dysgalactiae* GapC, *S. uberis* GapC, Mig, or *S. dysgalactiae* GapC plus Mig and then challenged by intramammary infusion of *S. dysgalactiae*.

These results confirmed our early observations on the ability of either the *S. dysgalactiae* GapC or Mig proteins to protect dry cows against the *S. dysgalactiae* challenge. Interestingly, the *S. uberis* GapC protein protected as well as the *S. dysgalactiae* homologue, confirming our hypothesis that there would be substantial cross reactivity between the GapC proteins of various Streptococcal species. When GapC and Mig were combined, we did observe immunological interference as was reflected by decreased serum antibody titers against these antigens compared to when they were administered individually. This is an issue that will need to be addressed during the next 6-12 months.

The *S. uberis* vaccine trial involved immunization of milking cows with the *S. uberis* GapC, *S. dysgalactiae* GapC, CAMP chimera, *S. uberis* GapC plus CAMP chimera, and a placebo followed by an heterologous challenge with *S. uberis* SU1. The SCC in all the non-challenged quarters were compared to the SCC obtained from the challenged quarters of each individual group. The results indicated that immunization with the *S. uberis* GapC, CAMP chimera or *S. uberis* GapC plus CAMP chimera resulted in a substantial reduction of the inflammatory response in the vaccinated animals as measured by a decrease in the somatic cell counts compared to the placebo (control) group as shown in the figure below.

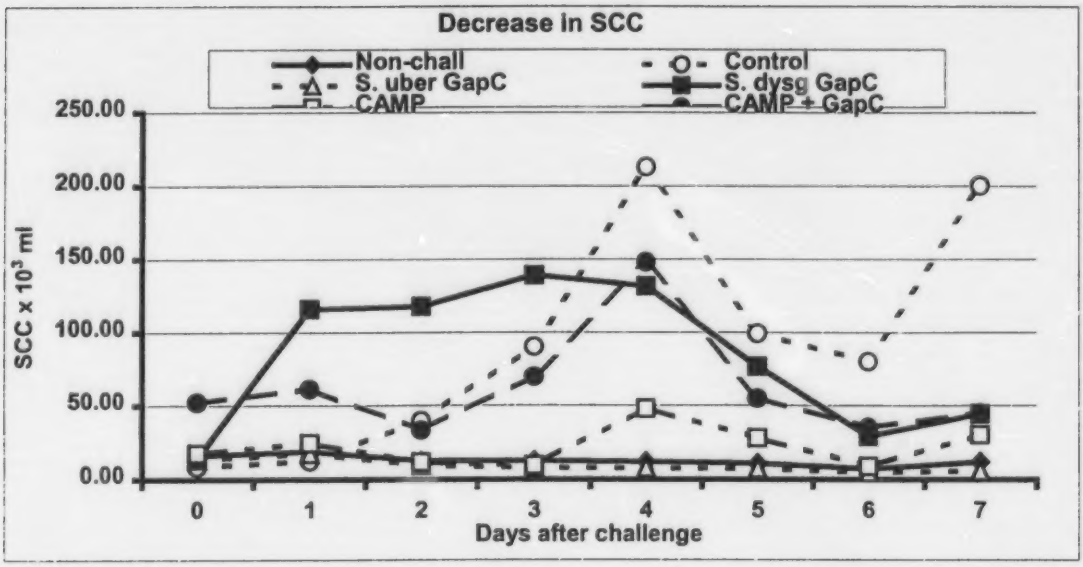


Figure 6. Protective capacity of *S. dysgalactiae* antigens. Somatic cell counts per quarter on seven days post-challenge. Animals were immunized with *S. dysgalactiae* GapC, *S. uberis* GapC, CAMP chimera, or *S. uberis* GapC plus CAMP chimera and then challenged by intramammary infusion of *S. uberis*.

To our surprise, the *S. dysgalactiae* GapC did not confer good protection against the *S. uberis* challenge despite the substantial cross-reaction between these two molecules. This is in contrast to the observation from the *S. dysgalactiae* vaccine trial in where the *S. uberis* GapC protein protected against the *S. dysgalactiae* challenge. At this moment we cannot explain these differences but these results emphasize the need for the chimera GapC with portions of the *S. dysgalactiae* and *S. uberis* GapC proteins contained in one single antigen. We did not observe immunological interference when the *S. uberis* GapC and CAMP chimera were combined in a single formulation.

The *S. agalactiae* vaccine trial involved immunization of milking cows with the *S. agalactiae* GapC, CAMP chimera, *S. agalactiae* GapC plus CAMP chimera, and a placebo. The immunized animals showed a high serum titre, but low milk titres against these antigens and the cows challenged with *S. agalactiae* showed signs of mastitis. This could be explained by the high challenge dose given to the animals or perhaps by the low antibody titre in milk.

In the last three months we have completed two more trials. The first trial was designed to determine the effect on the immune response to increasing amounts of *S. uberis* GapC in cows. The information collected in this trial will be used in the formulation of vaccines for successive trials. We immunized animals with 15, 40, and 100 ug of the GapC protein and measure the antibody levels at different times. The results are shown below.

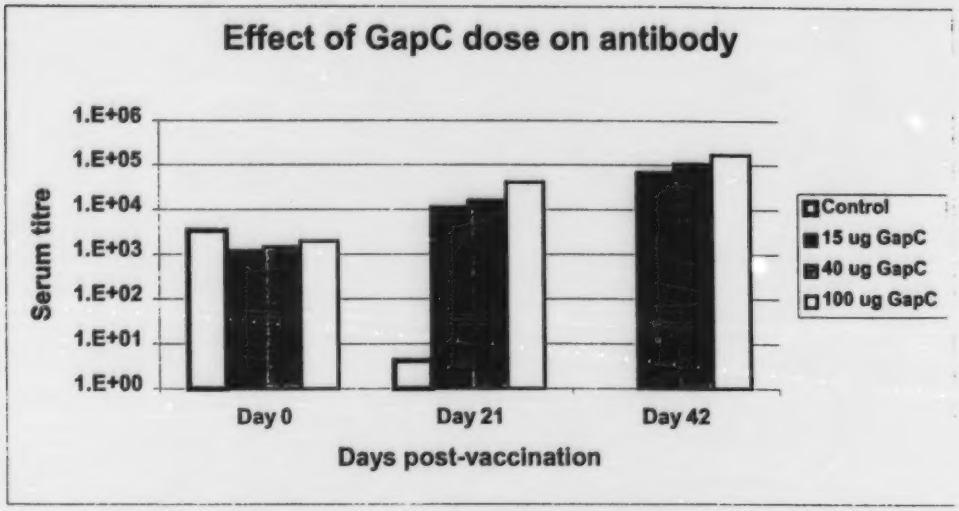


Figure 7. Serum antibody response to GapC. Serum antibody titres at days 0, 21, and 42 after immunization with increasing amount of *S. uberis* GapC protein.

The conclusion of this trial is that there is not significant differences between the antibody levels at day 42 using 15, 40 or 100 ug of GapC. These results suggest that as low as 15 ug of antigen might be enough to elicit a strong immune response. However before we decide on this amount of antigen, we need to measure the duration of the immune response as a function of the amount of GapC antigen injected into cows.

In the second trial we explored the immune response to the CAMP chimera when expressed on the surface of *Escherichia coli*. This was carried out to determine if specific antibodies against CAMP could be elicited when this protein was on the surface of *E. coli* and it is part of the studies leading to the development of a multi-functional vaccine against mastitis comprised of *E. coli*, streptococcal and staphylococcal antigens. The animals were vaccinated with a purified CAMP chimera, a detergent extract obtained from *E. coli* cells expressing CAMP on the cell surface, whole *E. coli* cells expressing CAMP on the surface, *E. coli* cells without the CAMP protein, and a placebo. The serum titres to the five antigens are presented below.

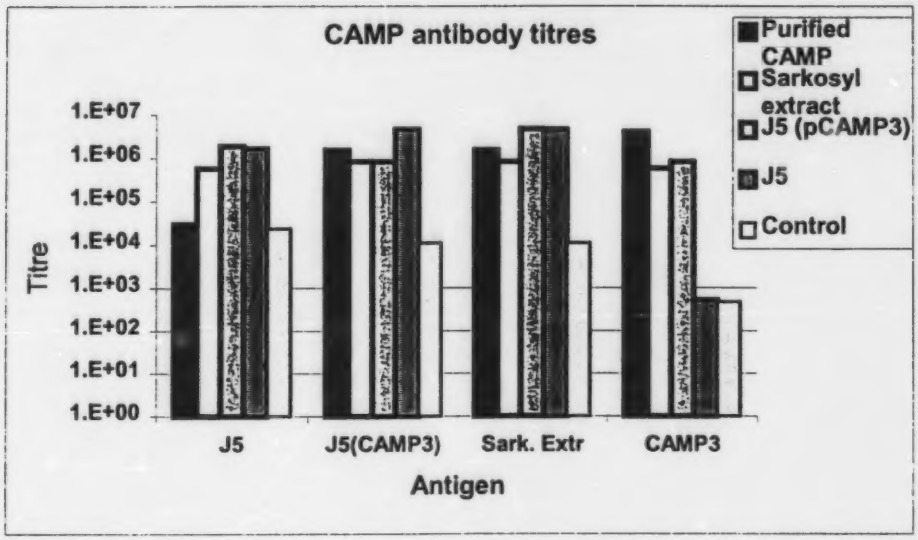


Figure 8. Serum antibody titres to CAMP chimera. Animals were immunized with purified CAMP, CAMP removed from the cell surface of *E. coli*, whole *E. coli* cells containing CAMP on the surface and *E. coli* whole cells, and a placebo. The serum titres to the different antigens is shown

These results indicated that animals vaccinated with either the detergent extract containing CAMP, or whole cells expressing CAMP on the surface possess a high serum titre against the purified CAMP product. This suggests that it is possible to express streptococcal antigens on the cell surface of *E. coli* and that these antigens will be recognized by the immune system of the animal.

These trials complete the proof of concept phase of this research project and now we are in the development phase with our commercial partner, Boehringer Ingelheim Vetmedica Inc.

3.3.4 Conclusions:

From these results we can conclude that we have identified antigens that when used as vaccines result in a protective immune response against mastitis caused by environmental streptococci. In addition, we have shown that it is possible to express streptococcal antigens on the cell surface of *E. coli* and that these antigens can elicit a strong immune response. These results will pave the way for the design of a multivalent vaccine to prevent mastitis caused by *E. coli*, *S. aureus*, and streptococci.

We have good information about the amounts of protein antigens to use in the vaccine formulations that would protect against infections. However, all these trials used vaccines prepared with one adjuvant and applied by only one possible route of immunization. In the next six months, vaccine trials are planned to determine the antigen-adjuvant formulation that would result in the highest immune response to the antigen. As the test antigen we will use the *S. uberis* GapC protein at the optimal amount indicated by the dose-response trial mentioned above and mixed with different adjuvants. The immune response to the antigen will be measured in the serum following subcutaneous vaccination and the antigen-adjuvant formulation that results in the highest immune

response to the antigen will be used in the following vaccine trial. In the second trial we will compare different routes of immunization for their ability to mount an immune response to the test antigen. The immune response to the antigen will be measured in the serum following subcutaneous and intranasal vaccinations or a combination of both. The immunization route that results in the highest immune response to the test antigen will be used for the next trial. For the third trial we will immunize lactating cows with the *S. uberis* GapC and the CAMP chimera antigens using the best antigen-adjuvant formulation and immunization route. The immune response to the antigens will be measured in the serum and milk.

3.3.5 Information of Benefit to Producers:

We have identified antigens that when used in vaccines can prevent mastitis caused by environmental streptococci and also started the studies leading to the formulation of a multivalent vaccine against the most common bacteria causing mastitis. In the next two years, we hope to complete most of the studies required for the application of a license to use and commercialize this vaccine.

3.4 Project Title: Subunit vaccines for *Haemophilus somnus*

3.4.1 Objectives:

The objective of this project is to develop a recombinant subunit vaccine for the prevention of hemophilosis in feedlot cattle.

3.4.2 Background and Approach:

H. somnus causes several disease syndromes in cattle, including reproductive and neurological disorders, pneumonia, arthritis, myocarditis, and others. During the past decade, the pattern of disease observed in Canadian feedlots has changed with a shift from the neurological form of the disease to pneumonia and infections of the heart. It is now the most economically important bacterial pathogen affecting feedlot cattle in Western Canada. Vaccines have been available for a number of years, but have had little impact upon the incidence of disease. VIDO developed a conventional *H. somnus* vaccine several years ago and we have been following this up to produce a second generation product which we believe will be more effective and compatible with existing management practices. The approach which we have used is to identify specific components produced by *H. somnus* which are essential for survival of this organism in the host. We have successfully characterized a number of these components, two of which have been shown to be protective in experimental vaccine trials. In addition, we have identified and characterized two stress proteins produced by the bacteria which are essential for its ability to cause disease.

3.4.3 Results:

Two protective antigens of *H. somnus*, LppB and TbpB, have been shown previously in our laboratory to protect animals against experimental infection. One of the goals of this research project was to develop methods for the production of these two protective antigens which would be compatible with production in a commercial setting. This work was initially done in collaboration with BIOSTAR Inc. and we successfully developed novel methods for the production of soluble antigen in both cases. Two vaccine trials were conducted with this antigen and we were able to demonstrate that the soluble proteins were as protective as their insoluble homologues which were tested previously. While this research was being carried out, BIOSTAR Inc. decided to continue with the production and marketing of the conventional *H. somnus* vaccine developed at VIDO and thus the rights to the product were transferred to Bayer Animal Health. Two years ago, Bayer restructured its animal vaccine division and decided not to focus on bovine respiratory disease vaccines and thus gave up the rights to the technology. We are now negotiating with the Alberta Research Council for an agreement in which they will produce material for international distribution.

Since *H. somnus* is capable of intracellular replication, a second objective of this project was to characterize two additional antigens, groEL and DnaK, which may be able to prevent intracellular multiplication. During 1997, a vaccine trial was conducted to test the protective capacity of these antigens. Groups of animals were immunized with each protein, alone and in combination and were subsequently challenged with virulent *H. somnus*. No differences were observed in clinical disease between immunized and unimmunized groups and thus it appears that these proteins are not

protective for *H. somnus*. Therefore, this line of work has been abandoned. These particular proteins have utility in vaccine production, as it has been demonstrated for groEL that coexpression of this protein with other recombinant antigens can increase protein solubility. Since the *P. haemolytica* vaccine produced by BIOSTAR Inc. was based upon inclusion bodies, we tested the ability of these proteins to drive antigen production from insoluble to soluble. While no significant differences were observed, we did observe that the DnaK protein was capable of enhancing the immune response to the *P. haemolytica* leukotoxin raising the potential of using this particular component as an adjuvant for use in other vaccines. This work is being pursued in combination with other vaccine development projects, including that for bovine mastitis.

3.4.4. Conclusions:

The objectives of this project, namely the development of a second generation *H. somnus* vaccine, have been successfully completed and the technology is currently being transferred to a Western Canadian fermentation group for further development and licensing. Thus, we anticipate that this vaccine will be available to producers in the near future.

3.4.5 Information of Benefit to Producers:

H. somnus has been increasing in prevalence over the past decade in Western Canada and the pattern of disease observed has shifted along with the changes in prevalence. It is now the most economically important pathogen affecting feedlot cattle in Western Canada. Vaccines which are currently available are all two dose products while those for other respiratory disease pathogens, such as *P. haemolytica*, are predominantly one dose vaccines. Thus, we have successfully developed a second generation *H. somnus* vaccine which should be compatible with one dose administration to feedlot cattle along with vaccines for *P. haemolytica* and other BRD pathogens.

3.5 Project Title: Vaccines for bovine rotavirus and coronavirus.

3.5.1 Objectives:

The **overall objective** of this project was to develop a recombinant subunit vaccine for the prevention of calf scours caused by rotavirus and coronavirus.

In order to complete the development of rotavirus and coronavirus subunit vaccines, the **specific objective** was to conduct a field trial using a combination recombinant vaccine containing antigens from three rotavirus serotypes and bovine coronavirus.

3.5.2 Background and Approach:

Among pathogens involved in neonatal calf diarrhoea, E. coli, bovine rotavirus, and bovine coronavirus are the major etiological agents. VIDO has developed a first generation vaccine for E. coli, rotavirus, and coronavirus, and for the past several years we have achieved significant progress towards the development of a second generation recombinant vaccine for the rotavirus and coronavirus components. We have identified major protective antigens for rotavirus and coronavirus. For rotavirus, the VP4 and VP7 proteins are the major protective antigens, and they comprise the outer capsid structure of the virion. The VP4 protein functions in hemagglutination, cell binding and cell fusion. We have cloned the VP4 gene of bovine rotavirus, and expressed a portion of it (VP8* subunit) in an E. coli expression system. The E. coli expression system is useful for production of large quantities of recombinant immunogens at low cost. For bovine coronavirus, we have identified the S glycoprotein as a major immunogen. The S protein forms a major spike on the surface of the virion, and contains hemagglutination, cell binding and fusion functions. As with the rotavirus VP8* protein, a portion of the coronavirus S protein gene (S2) has been cloned, modified, and expressed in E. coli. We have tested the recombinant rotavirus VP8* protein and coronavirus S2 protein produced in E. coli for their immunogenicity in cows. Neutralizing antibodies have been identified in colostrum of the immunized cows, demonstrating that these recombinant proteins are capable of eliciting high levels of colostral antibodies in pregnant cattle.

3.5.3 Results:

The VP8 protein of rotavirus and a portion of the S2 protein of coronavirus have been used to immunize pregnant cattle. The immune responses generated in colostrum and transferred to calves were shown to be as good or better than that produced with conventional vaccines. However, there are now three serotypes or subtypes of rotavirus, called P1, P5 and P11. Since the P1 subtype is present in the existing vaccine, the number of infections with the P1 subtype is lower than with the P5 and P11 subtypes. Therefore, in addition to VP8 of serotype P1, we have cloned and expressed the VP8 protein of serotypes P5 and P11 of rotavirus in E. coli.

Several vaccine formulations were subsequently tested in pregnant cattle in the field in order to assess their efficacy against rotavirus P1, P5 and P11, as well as coronavirus. Pregnant cows have been immunized twice with one of the following vaccine combinations: 1) S2 + VP8 (P1); 2) S2 + VP8 (P5); 3) S2 + VP8 (P11); 4) S2 + VP8 (P1+P5+P11); 5) Janecine commercial rota/corona vaccine; 6) Scourguard commercial rota/corona vaccine; 7) S2 + VSA3. Neutralizing antibody

responses to bovine rotavirus P1, P5 and P11, and coronavirus are currently being determined in the serum, colostrum and milk. Serum neutralizing antibody levels are also determined in the newborn calves.

Based on the samples tested thus far, VP8 of rotavirus serotype P1 and coronavirus S2 induced similar antibody titers as last year, confirming that the recombinant proteins are at least as effective as the currently available commercial vaccines. In addition, significantly increased titers specific for rotavirus serotypes 5 and 11 were induced following immunzation with VP8 of serotypes 5 and 11. This indicates that the recombinant VP8 of serotypes 5 and 11 is immunogenic in cattle, so that we can formulate a bovine rotavirus vaccine that will enhance immunity to the three most prevalent serotypes of rotavirus, P1, P5 and P11.

3.5.4 Conclusions:

1. The VP8* proteins of bovine rotavirus P1, P5 and P11, as well as the S2 protein of bovine coronavirus have been cloned and expressed at high levels as recombinant proteins in *E. coli.*

2. The recombinant VP8* (P1, P5 and P11) and S2 proteins induced neutralizing antibodies in pregnant cows, which resulted in significantly elevated antibody levels in the colostrum.

3. Very low cross reactivity occurred between VP8* (P1) specific antibodies and the P5 and P11 serotypes, so a rotavirus vaccine that is effective against the three common serotypes should contain VP8* from P1, P5 and P11 subtypes.

4. The VP8* and S2 subunit vaccines appeared to be effective, when administered as a combination vaccine with each other and/or with a commercial *E. coli* vaccine.

3.5.5 Information of Benefit to Producers:

The cattle industry in Canada, and more specifically in the Western provinces, accounts for a significant portion of the total farming industry, and one of the major risks faced by cattle producers is a substantial loss of animals or production due to infectious diseases. Neonatal scours in calves caused by *E. coli*, bovine rotavirus and coronavirus is one of the most common diseases, and the occurrence of outbreaks can cause significant economic losses. Commercial, killed vaccines against neonatal scours prepared by conventional methods are currently available in the market. However, these vaccines are not as economical to produce since neither rotavirus nor coronavirus grow in high quantities in cell culture. Furthermore, the current vaccines include only one rotavirus serotype, P1, which results in incomplete protection from infection with the other serotypes, P5 and P11. The development and use of a safe and effective vaccine for prevention of neonatal scours will reduce the disease losses and improve the efficiency of animal production, resulting in consumer savings. In addition, prevention of the disease by vaccination will help reduce the widespread use of antibiotics and other antibacterial drugs which can potentially cause meat residues and development of antibiotic-resistant bacteria. Effective prevention of the disease in cattle will also reduce costs for drugs, medication, veterinary services, and labour. Our recombinant vaccine is

produced with cost-effectiveness and confers broad spectrum protection against both bacterial and viral pathogens including heterotypic rotavirus infections.

Three groups will directly benefit from the information and technology that will be developed by our research. The first and most important group will be the cattle producers in Alberta and other Western provinces, who use the vaccine in their herds, and the second group will be the veterinary biologics manufacturing companies. The third group will be the consumers, who will benefit from improved meat quality and reduced prices.

3.6 Project Title: Vaccines for bovine herpesvirus-1.

3.6.1 Objectives:

The **overall objective** of this project was to develop a sensitive and specific ELISA, specific in order to differentiate gD-vaccinated calves from BHV-1 infected calves and sensitive in order to detect very low BHV-1 specific serum antibody levels.

In order to complete the development of a diagnostic test that differentiates vaccinated from infected animals, we had the following **specific objectives**:

1. Develop a single-dilution ELISA assay.
2. Compare the sensitivity of the single-dilution glycoprotein ELISA with a standard VN assay.

3.6.2 Background and Approach:

The ELISA that we developed is based on one of the major glycoproteins of BHV-1. Following exposure to and infection with BHV-1, calves produce serum antibodies that are primarily specific for the major glycoproteins present in the viral envelope. One of these glycoproteins is gD, which is used as a subunit vaccine. Four additional glycoproteins include gB, gH, gC and gE (previously called gI, gII, gIII and gE, respectively), each of which may be useful as a diagnostic antigen. Glycoprotein B has been selected as most appropriate for use in a BHV-1 diagnostic ELISA, on the basis of the following criteria: (a) BHV-1 infected and gD-immunized calves are clearly differentiated with this glycoprotein. (b) This glycoprotein induces an early and strong antibody response in BHV-1 infected or BHV-1 vaccinated calves. (c) The antibody response persists for a long time. (d) This glycoprotein has no or low cross-reactivity with sera from calves infected with unrelated pathogens.

Since the production of glycoproteins in BHV-1 infected cells is not high and therefore cannot be used on a production scale, the selected glycoprotein B was cloned and expressed in the high-level expression system that we currently use for the production of the gD subunit vaccine. In order to further enhance production levels and facilitate purification, the gB gene was modified, such that the glycoprotein is secreted (sgB) into the supernatant medium, instead of retained in the plasma membrane of the cells in culture.

3.6.3 Results:

a) Develop a single-dilution ELISA.

In order to make the ELISA more rapid and economical, we developed a single-dilution assay. In this assay, Immulon II 96-well microtiter plates are coated with 0.02 μg (optimum concentration) per well of sgB in 150 μl of coating buffer. After incubation at room temperature overnight, the plates are washed four times with PBST. Then, 50-fold dilutions of bovine sera in 200 μl PBST are added to the wells and the plates are incubated for 2 h at room temperature. On each plate there are 90 unknown serum samples and 6 control wells, three with positive serum (wells A1, A3 and H11) and three with negative serum (wells A2, H10 and H12). After four washes, the wells are incubated for 2 h at room temperature with a 1:15,000 dilution of alkaline

phosphatase-conjugated goat anti-bovine IgG (optimum conjugated antibody dilution) in PBST. Again, the plates are washed four times and then incubated with 150 µl of PNPP per well. After 1 h at room temperature, the color reaction in the wells is stopped with 50 µl per well of 0.3 M EDTA and recorded by reading the plates at 405/490 nm on an ELISA reader. The antibody levels (titers) are determined on the basis of the absorbance values (intensity of the color reaction) of the test sera, positive and negative control sera, included on each of the ELISA plates. The following mathematical equation format was used to calculate actual ELISA antibody titers:

1) $Sp = \frac{\text{(sample absorbance) - (avg. normal control absorbance)}}{\text{corrected positive control absorbance (CPC)}}$

2) Log 10 titer = (1.463 x log 10 Sp) + 3.197

3) Titer = antilog of log 10 titer

CPC = positive control absorbance - normal control absorbance

The positive/negative cut-off level was determined by calculating the mean titer plus three standard deviations, which was 152. Consequently, the cut-off value of the one-dilution gB ELISA was set at 200. To assess the specificity and sensitivity of the test, we analyzed negative sera and sera from BHV-1 infected or vaccinated calves. Of the 140 negative sera tested four had a titer >200, indicating a specificity of 97% and of the 186 positive sera tested eight had a titer of $\leq$ 200, indicating a sensitivity of 96%.

b) Compare the sensitivity of the one-dilution ELISA with a standard VN assay.

The glycoprotein-based single dilution ELISA should be one of the most rapid, sensitive and specific assays for the detection of BHV-1 specific antibodies in bovine sera, which also differentiates gD-vaccinated calves from BHV-1 infected animals. To confirm this and to determine whether this ELISA may be applied to routine diagnostic serological tests, we compared this assay with the most commonly used diagnostic serological assay, virus neutralization. A high correlation was observed between the sgB ELISA and SN assays.

3.6.4 Conclusions:

1. BHV-1 infected and gD vaccinated cattle can be differentiated with ELISA's based on individual glycoproteins of BHV-1, gB, gC, gE or gH.
2. Glycoprotein B was selected for further ELISA development and therefore, expressed and produced as a seereted form (sgB) in transfected MDBK cells in order to enhance production and facilitate purification.
3. A one-dilution sgB-ELISA was developed with 97% specificity and 96% sensitivity.
4. There is a good correlation between the sgB ELISA and VN assays.

3.6.5 Information of Benefit to Producers:

BHV-1 infections are responsible for severe economic losses worldwide. In North America, BHV-1 primarily causes respiratory infections and thereby is an initiator of bacterial pneumonia. This disease complex causes significant losses to the cattle industry, particularly to the feedlot operators in Alberta. BHV-1 may also be responsible for abortions. The strategy for control of BHV-1 in Canada has primarily involved vaccination with modified live or killed BHV-1 vaccines. Because of the limitations of these vaccines, we have developed a subunit gD vaccine, which is not only effective, but also provides the basis for differentiation of vaccinated from infected animals. Although some European countries have also applied extensive vaccination programs, other countries like Denmark and Switzerland have successfully eradicated BHV-1. In these countries, seropositive cattle were slaughtered, because they were assumed to be virus carriers. The Netherlands intend to soon start a vaccination-eradication program, using a "marked" vaccine, to differentiate vaccinated and infected animals.

At the present time, many countries only permit import of cattle and semen if the animals are seronegative for BHV-1. With more countries starting eradication programs, the availability of a specific and sensitive test for the detection of BHV-1 specific antibodies becomes vital for the export industry of cattle and semen in Alberta and North America in general. In addition, the availability of an effective "marked" BHV-1 subunit vaccine and a companion serological test to identify vaccinated animals would be beneficial to the Alberta cattle industry, at this moment, but perhaps even more so in the future in case Canada follows suit and initiates a BHV-1 eradication program.

While we developed this diagnostic assay for BHV-1, we obtained information about the kinetics, level and specificity of the BHV-1 specific antibody response after BHV-1 infection or immunization with BHV-1 conventional and subunit vaccines. These data provide information about the duration of immunity induced by natural infection and different types of BHV-1 vaccines. Secondly, a single dilution ELISA assay, which can now be commercially developed, is more rapid and cheaper than established methods for BHV-1 diagnosis, and therefore, may be useful for diagnostic laboratories. Finally, a diagnostic single dilution ELISA kit, which differentiates gD-vaccinated from BHV-1 infected cattle, may be very useful for epidemiologists studying the incidence of BHV-1.

3.7. Project Title: Development of bovine adenovirus as a live vaccine vector.

3.7.1 Objectives:

The main objective of this project is to develop bovine adenovirus as a live vector for the delivery of antigens from other bovine pathogens to mucosal surfaces of cattle.

Specific Objectives:

1. Construct bovine adenovirus strains containing genes from BRSV.
2. Characterize the role of E3 deletions in pathogenesis.
3. Characterize the E1 region, including proteins produced.

3.7.2 Background and Approach:

Subunit vaccines often do not induce the breadth of immunity produced by a natural infection (cell mediated and mucosal immunity) and they require strong adjuvants to elicit an immune response. Furthermore, the production of subunit vaccines for use in veterinary medicine is dictated by economics and it is not always cost effective to produce viral subunit antigens using recombinant DNA technology. Therefore, novel approaches are required to reduce the cost of immunization and to ensure that a broad spectrum of immunity develops. One feasible way of achieving this is to use a crippled virus to deliver viral antigens, and bovine adenovirus is an ideal candidate. Two regions of the virus, termed E1 and E3, have been identified in human adenovirus where foreign genes can be inserted, leading to attenuation. We are extending this work to bovine adenovirus and are characterizing these two regions using conventional molecular biology techniques and are also inserting foreign genes into both loci in order to produce vaccines which can be delivered to mucosal surfaces. We first inserted a marker gene coding for the luciferase protein in order to test the feasibility of this system and showed that the protein was expressed in infected cells and also when the virus was used to infect animals. Our approach is now to extend this work by inserting the genes coding for relevant antigens from viral pathogens including BRSV, BVD, and PI-3. Once these recombinant viruses are constructed, we will test the immune responses against the foreign proteins in cattle and also test these vectored vaccines for safety and efficacy.

3.7.3 Results:

a) Method of construction of recombinant BAV-3.

In order to have a simple and efficient method of constructing bovine adenovirus (BAV) recombinants, we constructed a full length bovine adenovirus 3 clone in *E. coli* by using the bacterial homologous recombination machinery in such a way that unique restriction sites were introduced immediately upstream of left ITR and downstream of right ITR. Unique restriction enzyme digested full length clone DNA when transfected in BAV3 E1 transformed MDBK cells produced viral plaques. The restriction enzyme analysis of virion DNA confirmed the identity of recombinant virus as wild-type. Our results suggest that the full length BAV-3 clone constructed in *E. coli* is infectious. Thus, we now have a very efficient method of introducing deletions / foreign genes in BAV-3 genome in a plasmid and then using plasmid DNA for isolating recombinant BAV-3.

b) Construction of E1 and or E3 deleted BAV3

E3 deletion. Earlier we had determined the sequence of E3 region of BAV3. In order to determine the true boundaries of E3 (which could help in deleting the appropriate ORF), we constructed a transcription map of E3 region based on S1 analysis, Northern blotting and cDNA sequencing. Our results indicated that five transcripts originated from the E3 region and major late promoter with the potential to code for four proteins (284R,121R,86Rand 82R). Appropriate portions of these predicted proteins were fused with GST protein and these fusion proteins are injected into rabbits to raise antiserum. Identification and characterization of these proteins suggested that 284R is expressed as a 46kDa glycoprotein in BAV3 infected MDBK which can also occur as dimer (94kDa). The 121R is expressed as a 15 kDa protein in BAV3 infected MDBK which is not modified by addition of glycans.

In order to determine their role in viral replication and pathogenesis, we started constructing the recombinant BAV3 with deletions in individual E3 specific proteins(based on transcriptional data)genes. We constructed a recombinant BAV3 containing a deletion in E3 121R proteins. Analysis of this recombinant showed that 121R (15 kDa) protein is not expressed in recombinant infected MDBK cells. Since 121R shows sequence homology to 14.7 K protein (protects cells from TNF destruction) of human adenovirus-5(HAV5), we sought to determine if 121R also can protect the cells from the TNF destruction. Our results suggest that 121R protein of E3 region of BAV3 can protect the cells from TNF lysis.

Next, we constructed a E3 deleted BAV-3 clone in *E. coli*. Using PCR, we first constructed a transfer plasmid (pE3d), in which 1.3 kb of E3 region is deleted and replaced by a unique restriction site (construction described in earlier report). Taking advantage of homologous recombination machinery of *E. coli*, this E3 deletion was introduced into full length BAV-3 clone by homologous recombination in *E. coli*. The BAV-3 E3 deleted genomic clone with correct restriction enzyme pattern was selected and the appropriate DNA was transfected into bovine cells. The cytopathic effects were visible in transfected bovine cells in 15 days. The recombinant BAV-3 was plaque purified and confirmed to contain the desired 1.3 kb deletion by restriction enzyme analysis and Southern blot analysis. A single step growth curve analysis suggested that the replication and growth characteristics of the recombinant BAV-3 are similar to wild type BAV-3 in bovine cells.

In order to determine the role of E3 region proteins in pathogenesis, initially we immunized cotton rats (small animal model) with recombinant E3 deleted BAV3. Our results suggest that E3 deleted BAV3 produces pulmonary lesions similar to those of wild type BAV3. Subsequently, we immunized calves intranasally with wild-type or E3 deleted BAV3. None of the animals showed any sign of clinical disease. Virus excretion was observed in one animal. In addition, significant rise in BAV3 specific IgG and IgA was observed in animals of both groups. This suggests that deletion of E3 proteins is not involved in increasing the virulence of BAV3.

E1 region. Earlier, E1 region of BAV-3 was identified and sequenced. In order to determine the exact boundaries of the open reading frames (ORFs) encoded by E1 region, we first sought to determine the transcriptional map of the E1 region. Our results suggest that 5 E1A and 2E1B specific transcripts originating from the region overlap and are 3' co-terminal with pIX transcript. In addition, E1 region extends from nucleotide 298 to nucleotide 3635. These transcripts has the potential to encode three main proteins named 220R, 157R and 420R. To help identify and characterize these proteins, we produced synthetic peptides as well as fused the coding sequence of

each protein in frame with GST protein. The individual peptides or fusion proteins were injected into rabbits to raise monospecific antisera against each E1 specific protein. Analysis of these proteins in BAV3 infected MDBK cells suggested while E1A (220R) encodes heterogenous related phosphoproteins (45 to 57kDa), E1b codes for two separate phosphoproteins of 19kDa (157R) and 48kDa (420R).

Since E1 region is essential for the viral replication, recombinant BAV-3 missing E1 region can only be isolated and propagated in cell lines expressing E1 proteins. To achieve this, two cell lines expressing E1 region proteins were isolated.

(i) 6.93.9 cell line. This cell line was isolated by transfection of MDBK cells with a plasmid containing E1 region of BAV-3 and NeoR marker. This cell line supported the growth of E1 deleted HAV-5 to 10^4 pfu/ml. However, repeated attempts to isolate E1 deleted BAV-3 using 6.93.9 cells proved futile.

(ii) VIDO R2. This cell line was isolated by transformation of fetal bovine retina cells by E1 region of HAV-5. This cell line supported the growth the growth of E1 deleted HAV-5 to 10^6 pfu/ml. In addition, we could also isolate an E1 deleted BAV-3 (described below).

To construct an E1 deleted BAV-3, we constructed a full length BAV-3 genome in a plasmid, which contained 540 bp deletion in E1A region and 1.2 kb deletion in E3 region. Transfection of VIDO R2 cells with this plasmid DNA produced cytopathic effects in 15 days. The identity of the replication defective BAV-3 was confirmed by restriction enzyme analysis of virion DNA. In addition, this recombinant named BAV500 replicated in VIDO R2 but not in fetal bovine retina cells. This confirmed that we have isolated a replication defective (E1-E3 deleted) BAV-3.

Next, we constructed full length BAV3 genome in a plasmid which contained insertion of a three way stop codon in $E1B^{small}$ region of BAV3. Transfection of VIDO R3 cells with this plasmid DNA produced cytopathic effects in 20 days. The identity of the recombinant BAV3 was confirmed by restriction enzyme analysis of virion DNA. Unlike E1A, the requirement of $E1B^{small}$ for replication of BAV3 was cell type dependent.

In order to determine the role of BAV3 E1A proteins in viral replication in vivo, we immunized the calves with wild-type or E1a-E3 deleted BAV3. None of the animals showed any sign of clinical disease. In addition, virus could be isolated only from animals inoculated with wild type BAV3. This suggests that BAV3 E1a is also required for BAV3 replication in vivo.

c) Construction of Recombinants Expressing Foreign Genes

Use of E3 region for expression of vaccine antigens

Initially, we constructed E3 deleted full length BAV3 genomic clones containing bovine herpesvirus-1 glycoprotein gD (full length or truncated) inserted inbto E3 region. Transfection of fetal bovine retina cells with either of the plasmid DNA produced recombinant virus. The identity of the recombinant virus was confirmed by restriction enzyme analysis of virion DNAs. Analysis of gD expression by these recombinant viruses suggests that the antigenicity, processing and transport of the recombinant gD (full length / truncated) protein is similar to the authentic gD expressed by bovine herpesvirus-1.

Next, we have constructed an E3 deleted full length BAV3 genomic clones containing bovine coronavirus HE glycoprotein gene with or without intron sequences at the 5' end. Recombinant viruses could be obtained when either construct DNA was transfected into bovine cells. The identity of the recombinant viruses was confirmed by restriction enzyme analysis of virion DNA. However recombinant HE protein could only be detected in the cells infected with recombinant BAV3 containing HE gene flanked by an intron at the 5' end.

We also constructed E3 deleted full length BAV3 genomic clones containing different forms of bovine viral diarrhea E2 glycoprotein gene inserted in E3 region. Transfection of fetal bovine retina cells with these plasmid DNAs produced recombinant viruses. The identity of the recombinant viruses was confirmed by restriction enzyme analysis of virion DNA and immunoprecipitation of E2 from virus infected cell lysates.

Finally we constructed E3 deleted full length BAV3 genomiuc clone containing G glycoprotein gene of BRSV inserted into E3 region. Transfection of bovine cells with this plasmid DNA produced cytopathic effects. The identity of the recombinant virus was confirmed by restriction enzyme analysisof virion DNA and immunoprecipitation of protein from recombinant virus infected cell lysates.

In addition, we have constructed several replication-competent recombinant BAV-3s expressing a reporter gene under the control of different promoters in E3 region. The identity of these recombinants was confirmed by restriction enzyme analysis of virion DNA and by immunoprecipitation of the reporter protein from virus infected cells. Our results suggest that duration and kinetics of foreign gene expression can be altered by using exogenous transcriptional elements.

Use of E1 region for expression of vaccine antigens

To determine if E1A region can be used for the expression of foreign genes, we inserted gD gene of BHV-1 flanked by SV40 promoter and polyadenylation signals in the E1A region of E1a-E3 deleted BAV3 genomic cone. Transfection of VIDO R2 cells produced cytopathic effects in 21 days. The identity of the recombinant virus was confirmed by restriction enzyme analysis of virion DNA and immunoprecip- itation of gD from recombinant virus infected cell lysates.

d) Immune responses

Since we have constructed recombinant BAV3 expressing full length or truncated form of bovine herpesvirus-1 glycoprotein gD, we first determined if immunization of animals with these recombinants leads to the induction of a mucosal immune response. We immunized cotton ratsintranasally twice (three weeks apart) with 10^7 PFU of different recombinant viruses. At different times post inoculation we collected samples serum, nasal and lung washes for analysisof IgG and IgA responses. Our results suggests that intranasal immunization of cotton rats induces both systemic and mucosal immune response against vaccine antigen (Fig. 1).

Subsequently, we immunized calves intranasally with gD or gDt expressing recombinants for assessing the efficacy of these recombinants in providing protection against bovine herpesvirus-1 challenge. Our results suggest that (I) both gD or gDt induced BHV-1 gD specific immune response of similar magnitude, (II) gD specific immune responses were induced even in the presence of high BAV3 specific antibodies in animals (Fig. 2) , and (III) recombinant (expressing gD or gDt) immunized animals showed significant differences in temperature, clinical signs, nasal

lesions, depression and virus shedding when compared with control animals (immunized with E3 deleted BAV3)(Fig.3).

We next determined the immunogenicity and efficacy of replication-defective (E1a-E3 deleted) and replication-competent (E3 deleted) expressing BHV-1 glycoprotein gD (full length). The calves were immunized intratracheally / subcutaneously with 10^8 PFU of recombinant or control virus (E1a-E3 deleted) and samples were collected for analysis of immune responses. Our results show that efficacy of replication-competent BAV3 to induce gD specific antibodies in the serum and the nasal secretions was superior to that of replication-defective BAV3. In addition, partial protection from BHV-1 challenge was induced in calves immunized with replication-competent BAV3. Our results suggest that intratracheal / subcutaneous immunizations with recombinant BAV3 may not be a promising strategy for immunization against BHV-1

We next determined the immunogenicity and efficacy of replication-competent (E3 deleted) expressing BVDV glycoprotein E2 (original full length or synthetic full length). The cotton rats were immunized intranasally with 10^7 PFU of either recombinant or control virus (E3 deleted) and samples were collected for analysis of immune responses. Our results show that replication-competent BAV3 expressing BVDV E3 (original or synthetic) induced E2 specific antibodies in the serum and the nasal secretions. However the magnitude of immune response was superior in animals expressing E2 under exogenous promoter.

3.7.4 Conclusions:

We have determined the feasibility of developing BAV3 as a vector for the delivery of vaccine antigens to the mucosal surfaces of cattle. Initially, we developed an easy method of constructing recombinant BAV3 using homologous recombination machinery of *E. coli*. We have identified different regions and constructed recombinant BAV3 deleted in E1 and / or E3 region, which are avirulent when administered in animals and develop vector specific IgG and IgA responses. We have demonstrated that E1 or E3 regions can be used for the exression of vaccine antigens of DNA and RNA viruses. Finally, we have provided the proof of concept of using BAV3 as a vector by demonstrating that intranasal immunization of calves with recombinant BAV3 expressing gD glycoprotein of BHV-1 induces protective immune responses against BHV-1 challenge in cattle. These studies have formed the foundation for establishing a family of safe and effective cattle vaccines using bovine adenovirus as a vector.

3.7.5 Information Of Benefit To Producers:

Significant economic losses (mortality, loss of productivity, treatment costs etc.) in the cattle industry are caused by infection. Developing effective preventive measures such as vaccination could prevent these losses. The results of this study suggest that bovine adenovirus-3 can be used as a live viral vector for the delivery of vaccine antigens to the mucosal surfaces of cattle. This unique approach of producing new generation of safe and efficacious vaccines should help in greater economic benefits. The intranasal route of immunization will prevent not only the meat quality damage (due to intramuscular injections) but will also help to immunize calves at birth (circumventing the problem of passive immunity). In addition, availability of recombinant BAV-3 expressing vaccine antigens is helping to evaluate other routes of immunization (e. g. Oral route). If successful, oral route of immunization will help in reducing the handling of animals thus addition more economic benefits.

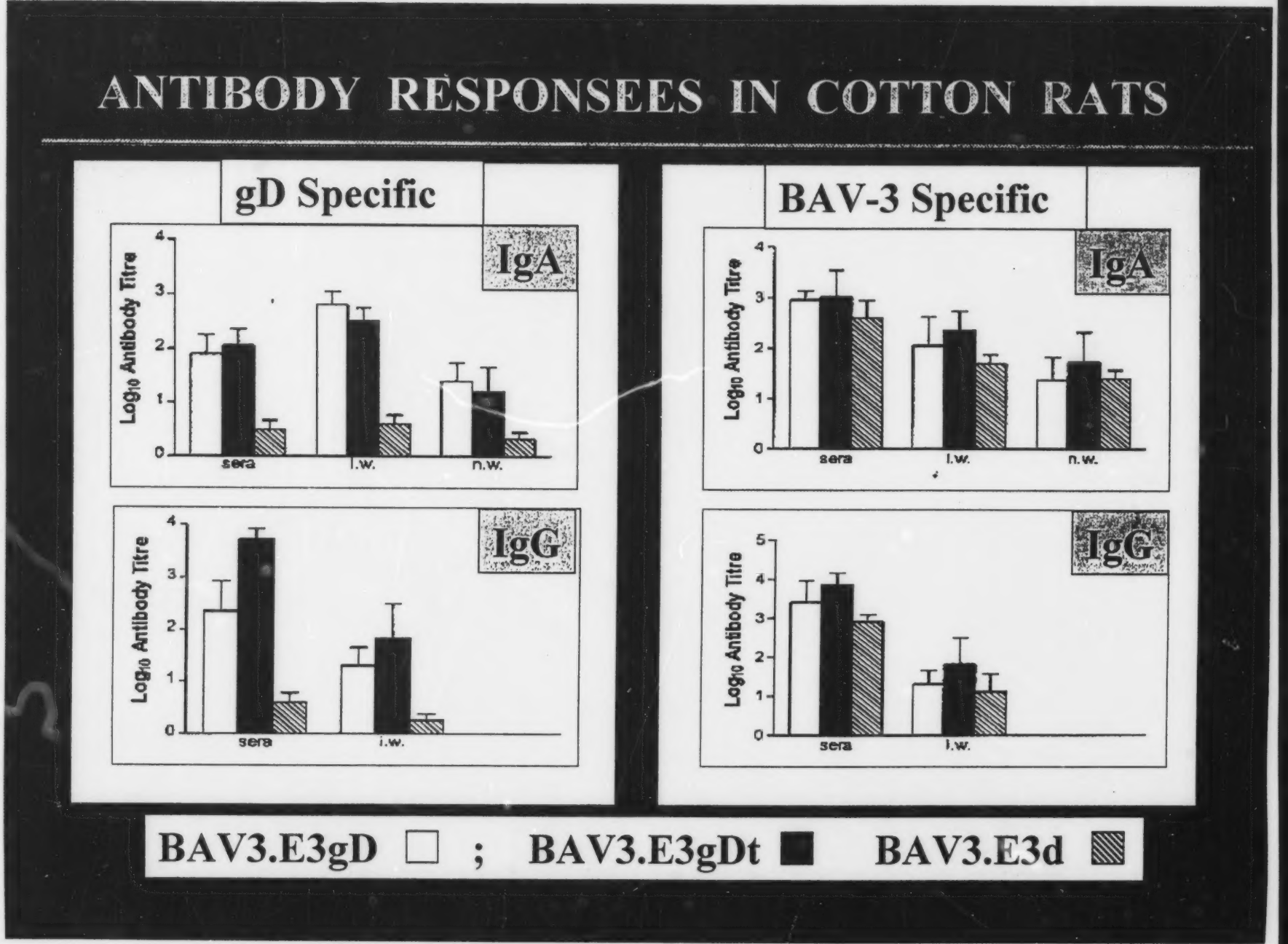
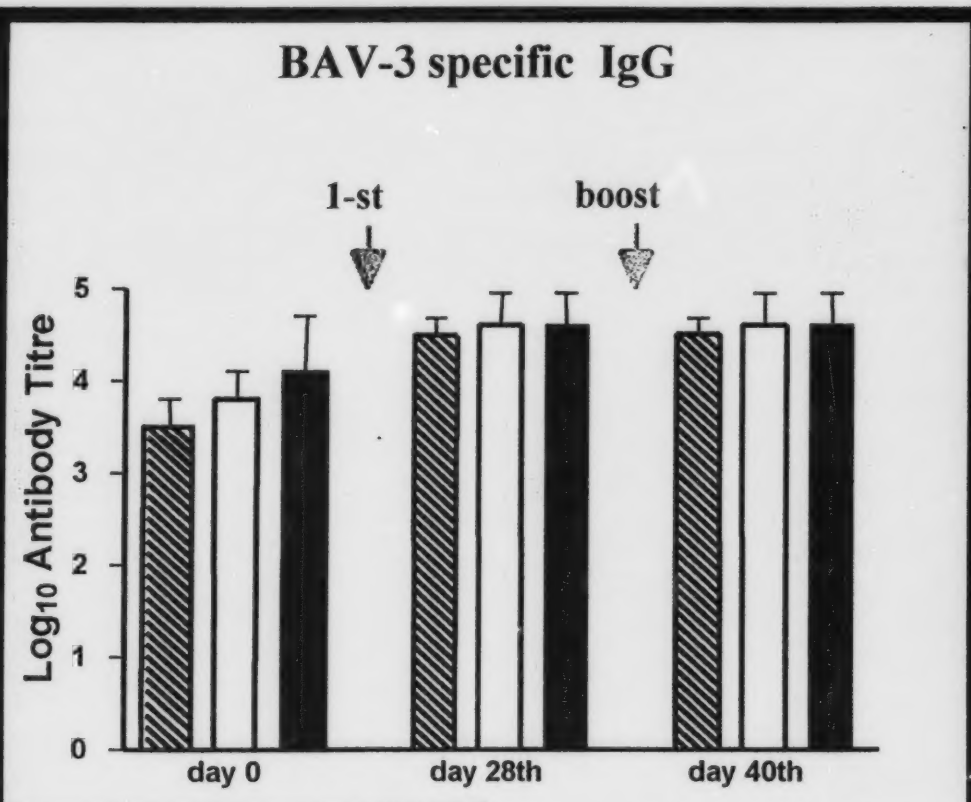
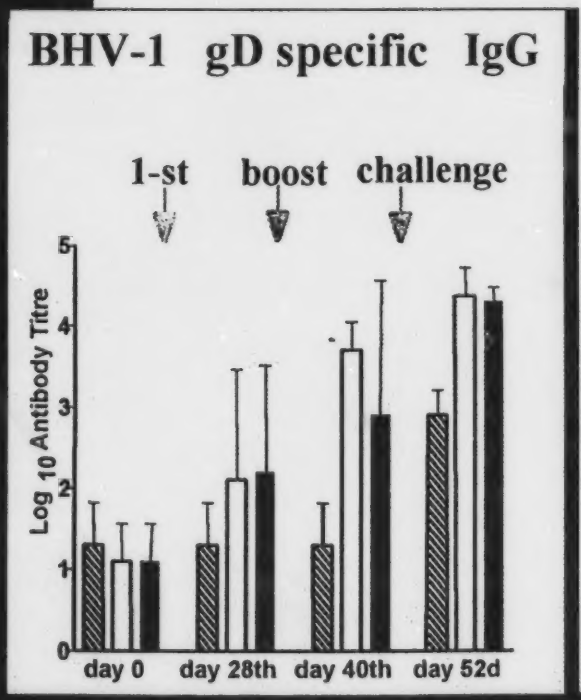

ANTIBODY RESPONSEES IN COTTON RATS
gD Specific
BAV-3 Specific
IgA
IgG
Log10 Antibody Titre
sera
l.w.
n.w.
BAV3.E3gD ; BAV3.E3gDt BAV3.E3d

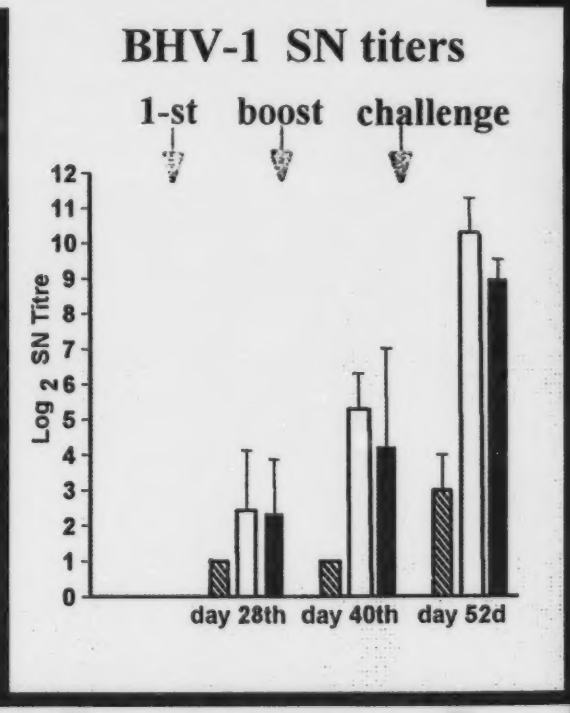
Figure 1

Figure 2

ANTIBODY RESPONSES IN SERA IMMUNIZED CALVES







BAV3.E3d ▨; BAV3.E3gD □; BAV3.E3gDt ■;

EFFECT OF IMMUNIZATION ON CLINICAL DISEASE IN CALVES CHALLENGED WITH BHV-1

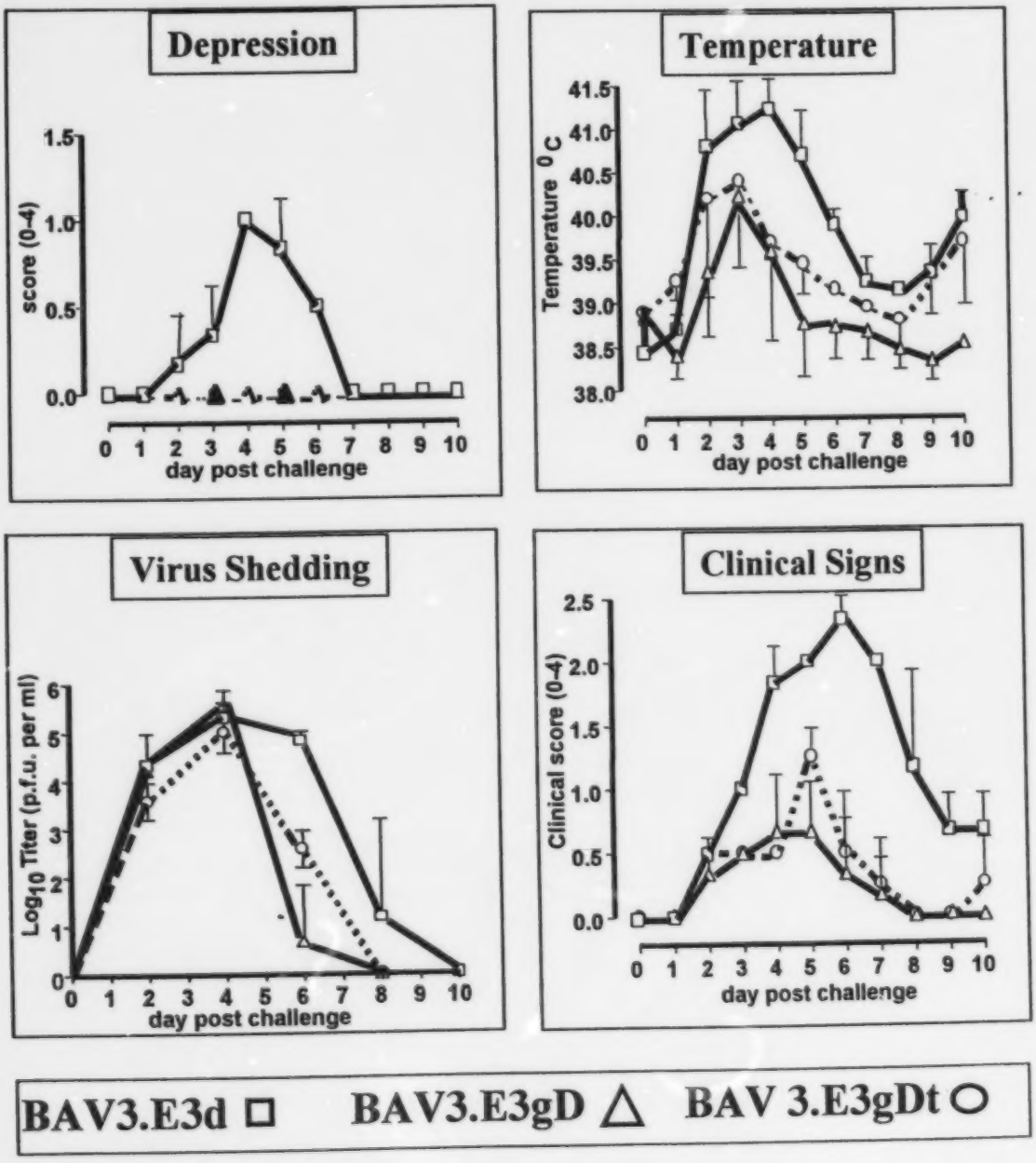


Figure 3

3.8 Project Title: DNA Immunization

3.8.1 Objectives:

The **long-term objective** of this project is to develop nucleic acid immunization technology for use in food-producing animals. The **specific objectives** were to:

1. Develop methods to increase the level of antigen production
2. Test the ability of cytokines to modulate the immune response following DNA immunization.
3. Optimize intradermal and intranasal delivery of plasmid DNA.
4. Develop methods for efficient epidermal delivery of plasmid DNA.
5. Identify optimal parameters for gene gun mediated delivery of DNA vaccines to cattle.
6. Assess the duration of immunity in cattle following DNA imunization
7. Identify CTL responses to BHV-1 following immunization.
8. Determine whether multiple genes can be co-administrered as multivalent vaccines.
9. Carry out efficacy testing using combination vaccines in cattle.

3.8.2 Background and Approaches:

DNA vaccination represents a new and potentially powerful approach to the development of vaccines and is accomplished by injecting purified genes coding for protective viral or bacterial antigens directly into the animal, which is then followed by uptake and expression of the DNA in host cells. Subsequent expression of protein in the host cells results in the presentation of normally processed or modified forms of the protein to the immune system of the animal. The use of pure plasmid DNA offers many advantages over other delivery systems. These include greater ease and speed of production, easier quality control, non-integration of the DNA and lack of immunogenicity of the vector itself. Further benefits of this method of immunization include longevity of the immune response, the potential of neonatal immunization, induction of a broad spectrum of immune responses and immunity to a variety of pathogens through the use of multivalent vaccines.

Experiments in laboratory animals like mice can provide very useful information regarding the effect of antigen form and methods to modulate the immune response. However, it has been much easier to induce immune responses in mice than in most other species, specifically by intramuscular (IM) inoculation. Therefore, it is important to also use a large and more relevant animal model to address questions concerning dose, specific immune responses, route and method of delivery. The purpose of our study was to optimize this approach for vaccination of cattle, using an intradermal (ID), epidermal (ED) or intranasal (IN) route of immunization.

Previously, we have identified the genes that code for the proteins responsible for the induction of protective immunity against a number of important respiratory and enteric infections in cattle, including bovine herpesvirus-1 (BHV-1), bovine parainfluenzavirus-3 (BPIV3), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), *Pasteurella haemolytica* and *Haemophilus somnus* (*H. somnus*). By using DNA immunization as an approach to vaccinate animals, the genes encoding these protective antigens can be directly delivered to the animals inside

plasmid vectors, such that the animals produce these proteins in their cells, and develop an immune response.

3.8.3 Results:

a) Develop methods to increase the level of antigen production.

Since with our first constructs relatively large amounts of DNA were needed to induce immune responses, specifically in cattle, one of our goals was to enhance foreign gene expression in order to increase the level of antigen production. First, to increase efficiency of DNA production in the laboratory, we replaced PBR322 with a pSL301 backbone, which resulted in 5- to 10-fold enhanced DNA yields from bacterial culture.

Expression of foreign genes in the laboratory and in the animals is driven by promoters, which may be weaker or stronger. For our previous experiments, we used the Rous Sarcoma LTR promoter-enhancer to drive expression of gD. Other promoters, like the mouse cytomegalovirus (MCMV) immediate early protein (IE) gene promoter have been shown to be stronger in several systems, so we replaced the RSV LTR with the MCMV IE promoter. In an attempt to increase mRNA stability, we also generated constructs with an extended SV40 polyadenylation signal. Although the addition of a longer polyadenylation signal did result in slightly higher responses in mice, we observed no difference between the RSV and MCMV-based promoter constructs in mice or cattle.

Therefore, we further pursued this objective by using the human cytomegalovirus (HCMV) immediate early protein (IE) gene promoter, enhancer and intron A for transcription initiation and the BGH terminator for transcription termination and polyadenylation. By using this system, we observed much higer levels of gD and tgD expression in tissue culture cells and stronger immune responses to the tgD gene both in mice and in cattle. More significantly, we also used the HCMV-IE intron A promoter to express two of the genes of bovine parainfluenza 3 (BoPI3) virus and observed immune responses that were 100 to 1000-fold higher than with the RSV LTR constructs.

b) Test the ability of cytokines to modulate the immune response following DNA immunization.

For a vaccine to be effective, it should not only induce enhanced, but also the appropriate type of immune responses in vaccinates, which may vary with different infectious agents. We have utilized the mouse model to study the effect of co-administration of cytokines on the level and type of immune response to a sub-optimal dose of tgD. Preliminary experiments showed that four cytokines had an effect on the immune response to tgD. IL-4 significantly increased the mean gD-specific antibody titer and this increase was maintained for at least six weeks. GM-CSF increased the number of mice that seroconverted after immunization and affected the kinetics and magnitude of the gD-specific antibody response, such that the mice responded earlier and stronger. IL-6 had a moderate up-regulatory effect on the magnitude of the tgD-specific antibody response. Although the magnitude of the immune response was not affected by co-administration of IFNγ-encoding plasmid, the antibody isotype and Th (Thelper) cell type was switched from Th2 to Th1 type response. At the doses used in these experiments, IL-1, IL-2, IL-10 and TNFα had no significant effect. These data indicated that some injected plasmid encoded cytokines can enhance and direct the immune response in mice, at least under circumstances whereby one dose was administered IM to mice.

Since reports in the literature indicate that the effects of cytokine co-administration are dependent on a number of variables, like the route, dose and time of administration of the cytokine, a second experiment was conducted to determine the optimum dose for two cytokines, GM-CSF and IFNγ. Groups of ten C3H/HeN mice were injected once IM with tgD-encoding plasmid (50 μg) combined with different doses of the GM-CSF- or IFNγ-encoding plasmid (2, 10 or 50 μg). Serum ELISA titers were determined 4 and 6 weeks later. In this experiment only the 50 μg dose of GM-CSF-encoding plasmid appeared to have a significant effect on the magnitude of the immune response to tgD. In addition, at 50 μg per dose the IFNγ-encoding plasmid also significantly enhanced the immune response to tgD, whereas the 10 μg dose appeared to inhibit. This experiment confirmed the potential of several cytokines to have an adjuvant effect on the immune response to tgD-encoding plasmid DNA. However, the effect appears to be dependent on the doses used, so it is important to perform dose titrations both of the antigen-encoding plasmid as well as the cytokine-encoding plasmid to obtain an appreciation of the true potential of these cytokines.

These experiments demonstrate the potential of several cytokines to have an adjuvant effect on the immune response to gD-encoding plasmid DNA. Thus, in order to assess the effect of cytokine co-administration in a ruminant, we have chosen to focus on GM-CSF and IL-4. In addition to the effects of these adjuvants observed by us in mice, reports in the literature indicate that these cytokines have an up-regulatory effect on antigen presenting cells, specifically dendritic cells, which are thought to be essential for the efficacy of DNA vaccines. We have decided to use sheep as a model to test immune responses to DNA immunization, for several reasons. First, the immune systems of sheep and cattle are very similar and secondly, sheep are more readily available and more economic to use. Recently, we have cloned the gene coding for ovine GM-CSF, inserted it in the plasmid used for DNA immunization and confirmed that the gene is functional in tissue culture *in vitro*. In addition, we are now cloning and expressing the gene encoding ovine IL-4.

c) Optimize intradermal and intranasal delivery of plasmid DNA.

In order to identify the most effective route of delivery of plasmid DNA, groups of six mice were immunized with 1.5 μg of tgD-encoding plasmid DNA by injection IM in the quadriceps or ID in the tail. Immune responses were followed for 28 weeks by measuring antibody levels against tgD in ELISAs. The response from animals given plasmid DNA by ID delivery were superior to those generated by IM injection. Independent of the route of immunization the immune responses induced by 1.5 μg of plasmid expressing tgD were maintained throughout the course of the experiment, which was 28 weeks.

As an approach to intranasal delivery, groups of six mice were immunized ID or IN with different liposome formulations containing 5 μg of tgD-encoding plasmid DNA. Although three different formulations resulted in gD-specific antibody responses after ID injection, IN immunization did not result in any antibody response, so this approach was not further pursued.

d) Develop methods for efficient epidermal delivery of plasmid DNA.

According to reports in the literature the gene gun is found to be one of the most effective tools for delivery to date, reducing the amount of plasmid needed by 10-100-fold, so we used the gene gun to immunize mice and demonstrated that gene gun mediated delivery of as little as one 0.01μg dose of BHV-1 gD-encoding plasmid on gold beads (as compared to a minimal dose of 0.1 μg ID or 1 μg IM) into the epidermis of the abdomen results in detectable antibody responses in

mice. A further dose titration demonstrated enhanced antibody titers with 0.1 μg and high antibody levels with 1 μg. Based on these promising results, we then immunized sheep with 2.5 μg of plasmid encoding either gB or gD (as compared to 250 μg ID). After two immunizations, we observed antibody, as well as T cell responses to each of these antigens, which confirmed that this method of immunization is effective in large animals. The immune responses in these sheep were followed for a period of three months, which demonstrated that gD-specific humoral and cellular responses persist. In contrast, although gB-specific cellular immune responses persisted, antibody responses declined.

e) Identify optimal parameters for gene gun mediated delivery of DNA vaccines to cattle.

Since there only have been a limited number of reports of gene gun delivery in non-rodent systems, we decided to optimize this method in cattle in order to find the best delivery site(s) and conditions. For gene gun immunization, gold beads were coated essentially as described by the manufacturer. Different methods and different amounts of plasmid were used to coat gold beads with diameters of 1.6 μm (BioRad) or 1-3 μm (Sigma). The optimal protocol was as follows. One hundred microliter (100 μl) volumes of 1.6 μm gold beads in 50 mM spermidine and DNA (5 μg/mg gold) in ddH_2O were mixed together. While vortexing, 100 μl of 1 M $CaCl_2$ was added drop-wise to the mixture. The gold was allowed to precipitate for 10 min at room temperature, washed three times in 100% ethanol, and dissolved to 4 mg gold/ml of a 50 mM polyvinylpyrrilodine (PVP) solution in ethanol. This mixture was coated to the inside of teflon tubing, which was cut into ½ inch segments and then used in the gene gun.

A Helios gene gun (BioRad) was used for all immunizations. Because the optimal conditions for delivery may be dependent on both the helium pressure ($lbs/inch^2$: psi) and the thickness of the skin, a number of different sites were tested, including the neck (300/200 psi), hip (450/300/200), shoulder (450/300/200 psi), base of the ear (300/200 psi), lip (300/200 psi), caudal fold (300/200 psi), auxilary region (300/200 psi) and vulva (mucosa) (300/200 psi). Some of these sites may be expected to induce mucosal immunity. The conditions for delivery of the plasmid were 1.25 μg of DNA and 0.25 mg gold per shot.

The level of expression of GFP was assessed 6 and 24 hr after delivery of the gold beads by taking punch biopsies in the center of the shots, as well as off-center. For microscopy, the punch biopsies were placed in formalin. For toluidine blue staining, samples remained in formalin until processing, and for fluorescent microscopy skin plugs were kept in formalin for 2-3 h, washed in PBS, frozen in 30% sucrose and stored at –70 °C. Toluidine blue slides were prepared by the histology facility at the Western College of Veterinary Medicine, University of Saskatchewan. For fluorescent microscopy, tissues were sectioned frozen on a minitome (IEC) and viewed with the aid of a fluorescent microscope by two observers.

The following observations were made on the sites analyzed. In the lip, the gold particles did not penetrate, regardless of the pressure used to deliver the gold. Subsequent toluidine blue staining showed a thick layer of dead tissue, which explains the lack of particle penetration. However, gold particles penetrated all of the other tissues tested. By far the majority of the cells that expressed the GFP protein were keratinocytes or epithelial cells, whereas occasionally a dendritic cell was found to express GFP. This indicates that either the keratinocytes are the primary cells producing the protective antigen, which is then taken up and processed by antigen presenting cells such as dendritic cells, or the dendritic cells that express the antigen migrate quickly to the local draining lymph nodes where they initiate the immune response.

In the skin of the neck, at 300 psi large numbers of gold particles and an inflammatory response were observed in the center of the shot, whereas the expression of GFP was found off-center and superficially. In contrast, at 200 psi, no inflammation was observed, whereas there was stronger expression of GFP. The levels of GFP expression at 6 h and 24 h were comparable.

In the skin of the shoulder and the hip, at 300 or 200 psi there were gold particles as well as strong expression of GFP in the center of the shot, both in the top layer and deeper in the epidermis. Off-center there was some GFP expression in the top layer. Little or no inflammation was observed at these pressures and there was always much more GFP expression 24 hr after the shot than 6 hr afterwards. In the shoulder there was more inflammation and less GFP expression at 450 psi, whereas in the hip 450 psi was similar to 300 or 200 psi.

In the caudal fold, strong GFP expression was observed throughout the epidermis at 6 hr after the shot, both at 200 and at 300 psi. At 300 psi considerable inflammation was observed at 24 hr, and GFP expression was found very superficially in the epidermis, which started to slough off. At 200 psi, little inflammation was observed at 24 hr, and GFP expression was also superficial.

In the auxilary region, little or no inflammation was observed at 24 hr after plasmid delivery at 200 psi, whereas there were numerous cells expressing GFP. At 6 hr post delivery hardly any GFP expressing cells were detected and there was no inflammation. At 300 psi fewer GFP expressing cells were detected at 24 hr and none at 6 hr after plasmid delivery.

In the vulva mucosa, no inflammatory response was observed at 200 or 300 psi, expression of GFP was primarily in the center of the shot throughout the epidermis, and maximum expression occurred 6 hr after the shot. Protein expressed by plasmid delivered into this tissue would be expected to induce not only systemic, but also mucosal immunity.

At the base of the ear, a site chosen for common draining lymph nodes with the respiratory tract, there was strong GFP expression 6 hr after delivery, but at 24 hr extensive inflammation and very little GFP expression was observed, regardless of the pressure used.

It is undesirable to have an inflammatory response, as it appears to inversely correlate with GFP expression, so based on these observations the skin on the shoulder, hip or auxilary region at 300 or 200 psi, as well as the vulva mucosa, neck or caudal fold at 200 psi, appear to be good sites for immunization.

f) Determine the duration of immunity in cattle following DNA immunization.

One of the anticipated advantages of DNA vaccines is the potential for inducing long-lasting immune responses. This expectation was mainly based on studies by ourselves and others in mice that demonstrated immune responses to last for at least two years. The duration of immunity has not been studied in many other species. In a recent trial, we started to determine the duration of immunity induced to a gD DNA vaccine as compared to the gD subunit vaccine. BHV-1 negative Herreford calves were randomly allocated to vaccine groups. Group 1 was immunized with a gD DNA vaccine (12.5 ug with the gene gun), group 2 received a gD subunit vaccine (5 ug in VSA3, intramuscular) and group 3 was injected with placebo (saline). Six weeks later they received a second immunization. The calves were bled at the time of immunization, as well as at regular intervals after each immunization. Humoral immune responses were determined by antigen-specific serum ELISA's and cellular immune responses by antigen-specific proliferation of peripheral blood mononuclear cells. This experiment demonstrated that the gD plasmid generated immune responses of lower magnitude, but better duration as compared to the very effective gD subunit vaccine. The antibody response in animals immunized by gene gun was maintained for at least 18 weeks, whereas protein immunized animals showed reductions after 6 weeks. Plasmid DNA immunized

animals had elevated proliferative responses for at least 12 weeks, whereas animals immunized with protein vaccine showed enhanced proliferation for only 3 weeks. After a secondary immunization, animals immunized with tgD encoding plasmid with the gene gun showed an increase in serum antibody levels, a rise in proliferative responses and enhanced IFN-□ production. Although the serum antibody levels in tgD plasmid immunized animals rose slowly, they also appeared to be maintained for period of time 5 months. These immune responses were found to be of sufficient magnitude to protect cattle against challenge with BHV-1, which demonstrates the efficacy of gene gun based delivery of DNA vaccines to target species.

g) Identify CTL responses to BHV-1 following immunization.

Because of the ability of BHV-1 gD to induce protection from viral challenge when administered as a subunit vaccine, the gD gene has thus far been the gene of choice for all DNA immunization studies. Glycoprotein D induces high levels of T helper cells and neutralizing antibodies. However, it appears to not have any epitopes that are involved in induction of CTL. Therefore, this goal has not been further pursued with the gD-encoding gene. Recently, the repertoire of genes used for DNA immunization has been expanded with BHV-1 gB, as described in the subsequent section of the report. Based on reports in the literature, gB may have CTL epitopes and therefore may be a good choice for future studies on the induction of CTL after DNA immunization.

h) Determine whether multiple genes can be co-administered as multivalent vaccines.

One of the anticipated advantages of DNA vaccines is the potential for development of multivalent vaccines, because all of the plasmids are prepared in the same solution, saline. Two of the protective antigens of BHV-1 are the gB and gD glycoproteins. In addition to BHV-1, several other pathogens contribute to bovine respiratory disease, including bovine respiratory syncytial (BoPI3) virus. The HN glycoprotein is one of the major protective antigens of BoPI3 virus. We have cloned the genes coding for BHV-1 gB and BoPI3 virus HN, and inserted them into the plasmid routinely used for DNA immunization. When the plasmids with the gB and HN genes were tested in tissue culture *in vitro*, gB and HN proteins were expressed, so the plasmids are functional.

In order to test whether multiple genes can be co-administered, we used the mouse model. First, we established that plasmids encoding gB and HN can induce immune responses, when injected into mice. These experiments demonstrated that like for gD, ID injection of genes encoding gB or HN results in high antibody levels. Subsequently, mice were immunized ID with the following combinations of genes: gB + gD; gD + HN, and gB + HN. Antibody responses were measured and, typically, all mice developed antibodies against each of the antigens, regardless of whether the genes were mixed or not. The immune responses to the gB and gD genes were absolutely not affected by the presence of the other genes. However, the magnitude of the immune response to the HN gene appeared to be consistently somewhat affected by the presence of the gD gene. Since this effect was not significant, we anticipate to be able to eliminate this effect by adjusting the doses administered.

Subsequently, we assessed the effect of mixing plasmids encoding different protective antigens into one formulation on the type of immune response induced. For this experiment we used plasmids encoding BHV-1 gD or influenza haemagglutintin (HA), because plasmid encoding BHV-1 gD induces a primarily humoral response, whereas influenza HA elicits a primarily cellular response under the conditions used. When plasmid encoding influenza HA was mixed in with BHV-

1 gD plasmid, the gD-specific immune response was not affected. However, when BHV-1 gD plasmid was added to the HA encoding plasmid, the immune response to HA shifted from primarily cellular to mainly humoral. This experiment demonstrated that under certain conditions the addition of a second plasmid can convert a primarily cellular, Th1-type, immune response to a mostly humoral, Th2-type, response. The reverse situation, however, was not found. These findings have important implications for the development of multivalent vaccines, specifically, where one particular type of response is desired.

i) Carry out efficacy testing using combination vaccines in cattle.

Since all plasmids are prepared in the same solution, another advantage of DNA immunization is the potential for the development of vaccines with multiple components. We demonstrated earlier in mice that although under certain conditions, there may be some interference due to mixing of plastids in one formulation, the magnitude of the immune responses induced to the individual components was not significantly affected. Recently, we have immunized three groups of BHV-1 and *Haemophilus somnus* negative calves with 1) a combination of plasmids encoding BHV-1 gB, gC and gD; 2) a combination of *H. somnus* LppB and DNAK, and 3) placebo. The animals were injected intradermally with 500 ug of each plasmid or saline for the placebo group. Four weeks after the first immunization they will receive a second dose of vaccine. The calves were bled at the time of immunization, and will be bled at regular intervals after each immunization. Humoral immune responses will be determined by antigen-specific serum ELISA's and cellular immune responses by antigen-specific proliferation of peripheral blood mononuclear cells. Depending on the level of immunity induced, the animals may be challenged with BHV-1 and *H. somnus*.

3.8.4 Conclusions

1. As an approach to start testing multiple plasmids in one DNA vaccine formulation, several constructs were generated with genes from different bovine respiratory pathogens. DNA immunization was first successfully used to immunize with genes from BHV-1, and subsequently this approach was also effective with genes from BPIV3.
2. A comparison of different routes of delivery demonstrated that for the viral genes tested, the ID route is superior to the IM route in mice and in cattle. This is an important observation not only from a viewpoint of enhanced immunity, but also in light of the current emphasis on meat quality. Furthermore, the dose of plasmid needed to induce immune responses may be further reduced by using gene gun mediated delivery.
3. Two doses of gene gun administered tgD encoding plasmid DNA induced immune responses of long duration, both in mice and in cattle.
4. Compatibility studies with genes from BHV-1, BPIV3 and influenzavirus indicated that, although under certain conditions interference may occur, plasmids generally may be mixed in one vaccine formulation without affecting the efficacy of the individual components.
5. In conclusion, significant progress has been made in the area of delivery of DNA vaccines to large animals. However, it remains one of the major issues before this technology can find widespread use in humans and animals, so this will continue to be an active area of research. Finally, additional combinations of plasmids in one vaccine formulation, as well

as the duration of the immune responses induced with DNA vaccines, will be tested in future experiments.

3.8.5 Information of Benefit to Producers:

The development of intradermal or epidermal delivery methods enables us to induce immune responses in ruminants with plasmid DNA that is delivered without adjuvant into the skin. This approach to vaccination should eliminate meat loss due to adjuvant residues or needle damage.

Furthermore, by delivering the DNA directly into the cells of the animals with a biolistic device or "gene gun", the amount of DNA needed to induce immunity is very low, which makes DNA vaccines cost-effective for veterinary use. Although we use respiratory pathogens as our model, this technology should eventually be applicable for development of a variety of vaccines. More than DNA vaccine can be used in one formulation, which is promising for the development of multivalent vaccines.

The results of our experiments also show that DNA vaccines can induce long-term immunity. Finally, as DNA vaccines are easy to produce, purify and manipulate, ultimately the expenses incurred by vaccination and treatment should be low, so producers should benefit significantly from using this method of vaccination.

3.9 Project Title: Measurement of host status

3.9.1 Objectives:

The overall objective of this project was to develop rapid, accurate, and cost-effective methods for monitoring disease in cattle. By focusing our attention on the animal and measuring its response to infection, rather than the presence of the causative organism, we hoped to develop tests capable of detecting a variety of diseases early in their development. Thus, the specific objective of this project was to develop tests to measure aspects of the early inflammatory response that will be of diagnostic and prognostic value.

The specific aims of this project were:

1. Assess the significance of measurement of haptoglobin concentration in different disease and health management situations.
2. Develop other tests to monitor the acute phase response.
3. Improve test formats to make them more practical for field use.

We looked at what information could be gained, and what decisions could be made, based on haptoglobin testing of cattle on arrival, or at the time of treatment, in the feedlot. We conducted two large field trials, and also retrospectively analysed two studies for which we had stored serum samples. In all studies, there was a significant association between elevated haptoglobin and disease. However, the strength of this association was not so great that haptoglobin testing could be used confidently as the method for detecting disease or basing a treatment decision. We also looked at the haptoglobin response to procedures such as vaccination, antibiotic injection, and castration. All of these common procedures induced haptoglobin production, and this response may interfere with detection of disease. Alternatively, this response could be used to assess the degree of tissue reaction to drugs or vaccines. Concurrent with the assessment of haptoglobin testing, we collaborated with a biotechnology firm to develop a prototype "cowside" test. From a few drops of blood, the test provides a positive/negative result in 5 minutes. To improve our ability to monitor the inflammatory response, and hopefully increase our diagnostic ability, we produced reagents for another acute phase reactant, interleukin 6.

3.9.2 Background and Approach:

Infections in cattle result in decreased productivity for the producer and increased suffering for the animal. As well, they contribute to reduced safety and wholesomeness of beef, either directly or consequent to the overuse of antibiotics. The concept of quality assurance throughout the beef production cycle is gaining favour in the industry. Tests that could monitor infection in growing animals and in animals destined for slaughter could be an integral component of the process to ensure a quality product to domestic consumers and to increase export potential.

Infectious disease is, by definition, the consequence of infection by microorganisms, such as bacteria, viruses, or parasites. However, the manifestations of clinical disease are a result of both the infecting organism and the animal's response to the infection. A common example of the animal's response to infection is a fever. At VIDO, we have been investigating the animal's response to various infections, and we have identified a number of reactions in addition to fever that occur. These include rapid non-

specific changes, common to many types of infections, collectively known as the acute phase response. Thus, we are studying the acute phase response of cattle to determine which features may provide a more accurate and rapid means of detecting infection. Haptoglobin is a protein, found in the blood of cattle, which is a marker for the acute phase response.

If fast, accurate, and inexpensive methods can be developed to monitor the acute phase response, then production losses due to infectious disease can be reduced. The proposed research could improve management of infectious diseases by: 1) implementing timely treatments based on accurate disease diagnosis, 2) reducing unnecessary treatment of animals which are not sick, or which are beyond salvage, and 3) providing a tool to monitor the effect of certain management practices on the animals. This would result in reduced losses to disease and death, more discriminate use of antibiotics, better animal husbandry, and a safer and a more wholesome product for domestic and export markets.

3.9.3 Results:

a) Use of Haptoglobin testing in feedlot cattle.

Use of Serum Haptoglobin Concentration to Detect Disease and to Predict Outcome in Feedlot Calves with Bovine Respiratory Disease

The objective of this study was to determine if a test for haptoglobin could discriminate between healthy calves and calves selected for treatment for shipping fever in a large commercial feedlot. As well, we wished to determine if the level of haptoglobin could predict the severity of disease and response to treatment.

Serum samples were collected daily from feedlot calves at a large Saskatchewan feedlot from Nov. 1 to Dec. 21, 1995. All calves were spring born, fall weaned and had recently entered the feedlot. Any calf that was run through the treatment chute during this time period had a serum sample collected and a complete feedlot health history recorded. Serum samples were stored at -20 C until analyzed for haptoglobin concentration by ELISA. A total of 881 samples were collected. Calves were brought to the treatment chute for a variety of reasons, including bovine respiratory disease (BRD), Haemophilosis, castration complications, miscellaneous problems, and some calves brought in as healthy calves for sampling (Table 1)

Table 1.

Reason for Sampling	Number of Cases
BRD	202
Haemophilosis	8
Castration Complications	17
Miscellaneous	29
Healthy	24
Total	280

For the sake of analysis these categories were collapsed into Shipping Fever (BRD and Haemophilosis), Other (Miscellaneous and Castration complications) and Healthy calves. Shipping Fever cases, which included 11 of the 12 mortalities in this study, had significantly higher haptoglobin levels than the Healthy group ($p<0.05$)(Fig. 1). However, even within this group there was considerable variation in the haptoglobin levels.

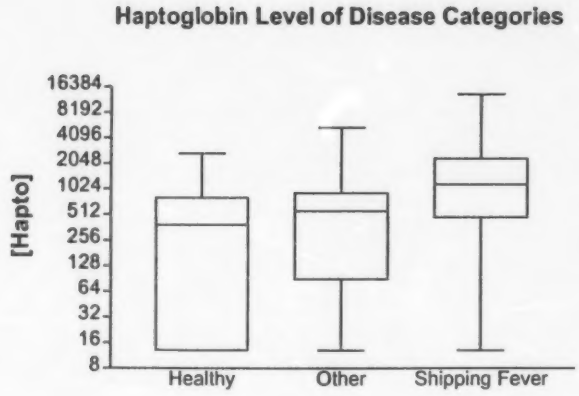


Figure 1.
Blood samples taken from calves at the time of treatment were analyzed for serum haptoglobin concentration. Results were categorized by reason for treatment, and are shown as median (line in box), 25th and 75th percentile (box), and range (bars). The median level of haptoglobin in shipping fever calves was significantly higher than that in healthy calves

Within the Shipping Fever group, some animals were treated even though they did not have temperatures of 40 C or greater, a level often used as a cut off for treatment. The calves with a temperature lower than 40 C had significantly lower haptoglobin levels (median = 842 ug/ml) than calves with temperatures => 40 C (median = 1258 ug/ml)) ($p<0.01$).

The shipping fever group was further categorized by outcome. Outcomes were divided into 3 levels, by examining the treatment record (Table 2). In the shipping fever group, 11 calves died of pneumonia or Haemophilosis.

Table 2. Outcome categories for Shipping Fever Cases

Outcome Level	Number of Pulls	Days Treated	Description
1	1	=< 3 days	Successful treatment after 1 treatment session of less than 3 days
2	=>2	> 3 days	Initial treatment unsuccessful, requiring prolonged treatment or a repeat treatment session.
3			Died of pneumonia or Haemophilosis

Using outcome levels (1,2 or 3), there was a trend towards increasing haptoglobin levels with increasing severity of disease, and the median level of haptoglobin in calves with outcome 1 was significantly less than that of calves which died ($p<0.05$)(Fig 2). If only calves with a fever are analyzed, then the median haptoglobin level of calves with outcome 1 was significantly less than those of calves with outcomes 2 and 3 ($p <0.05$).

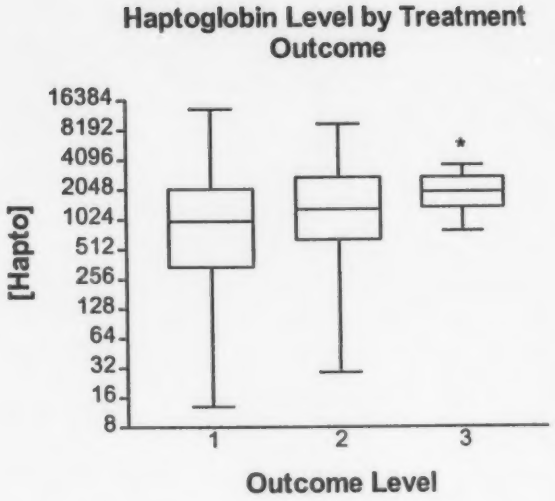


Figure 2.
Blood samples taken from calves at the time of treatment were analyzed for serum haptoglobin concentration. Results from Shipping Fever calves were categorized by outcome following treatment, and are shown as median (line in box), 25th and 75th percentile (box), and range (bars). The median haptoglobin concentration in calves that died was significantly higher than that in healthy calves. In calves with a fever, calves with outcome 2 or 3 were significantly greater then outcome 1

We had hoped that sampling throughout the treatment period would provide a more accurate reflection of the intensity of the acute phase response. However, haptoglobin levels tended to be highest on the day of initial sampling, and declined following treatment. The rate of decline of haptoglobin levels appeared to be less in cases that were more refractory to treatment.
Diagnostic tests are evaluated using measures of sensitivity, specificity, positive predictive values and negative predictive values. Sensitivity and Specificity are values of a test that are independent of the prevalence of disease in a population (see Fig 3). Ideally, the diagnostic test is compared to a Gold Standard or irrefutable reference standard diagnosis. In this study we must rely on the selection criteria of the feedlot cowboys for initial diagnosis, and the decisions of the treatment crew in determining repeat treatments (outcome level 2). These selection criteria and treatment decisions are obviously far from ideal. The most objective diagnosis is fatality (outcome level 3); however, many potentially fatal infections may have been successfully treated by prompt antibiotic therapy.

Any test that provides a continuous outcome measure such as haptoglobin can have any level set as the cutoff or diagnostic level. A value below this cutoff is test negative and a value above is test positive. The cutoff maybe set higher or lower depending on the relative impact of false positive and false negative diagnosis. In the feedlot an untreated case that results in fatality would result in a loss of hundreds of dollars. A treated non-case would result in the losses of tens of dollars. In other words, in the feedlot a false negative can have a cost impact of about ten fold greater than that of a false positive. Thus, the cutoff level for a feedlot test would likely be moved to increase sensitivity at the expense of specificity.

Ideally, if this test were to have utility in the feedlot it would be to reduce treatments without increasing the number of fatalities or repulls. Applying two tests together can usually increase the specificity of the diagnosis, if both tests must be positive before making a diagnosis. This test could be added to the current practice of observation and temperature to increase the specificity of the overall diagnosis. The diagnostic cutoff would have to be maintained at a low level (750) in order to avoid false negatives. Of the 150 calves that were treated for shipping fever with

temperatures equal to or greater than 40°C, 9 calves would not have been treated using the haptoglobin cutoff of 750. Of these 49 calves, only 6 required retreatment later and none of them went on to die. Since this could represent a substantial savings in treatment, it could justify a chute side test kit that was sold for 1 to 2 dollars a test. Unfortunately, it can be argued that these calves may not have done as well if they were not treated with antibiotics. The only way to confirm the benefit would be to develop the test kit and design a trial to test it in the field.

Retrospective Study of Haptoglobin Levels in Cattle on Entry to the Feedlot

The objective of this study was to determine if haptoglobin levels in calves at the time of entry into the feedlot were associated with the incidence of disease during the first 5 days in the feedlot.

A total of 5129 calves were in the trial, of which 4493 had their rectal temperature recorded, and 1007 had a blood sample taken on arrival. Of the calves for which a blood sample was available, 100 had a fever (>=40.5 C) on arrival, or were pulled for treatment during the first 5 days in the feedlot (Note that some calves that had a fever and were treated on arrival, also were treated on a subsequent day). As well, samples from an equivalent number of calves which had no fever on arrival and were not treated in the first 5 days, and were randomly distributed among the different lots, were tested (n=144). Note that all calves were given prophylactic antibiotic treatment (long-acting oxytetracycline) on arrival; in this report, treatment refers to the therapeutic administration of antibiotic (tilmicosin).

The serum haptoglobin concentration was determined for calves at the time of entry to the feedlot. These results were then categorized based on the treatment history of the calf into three groups (Fig 3).

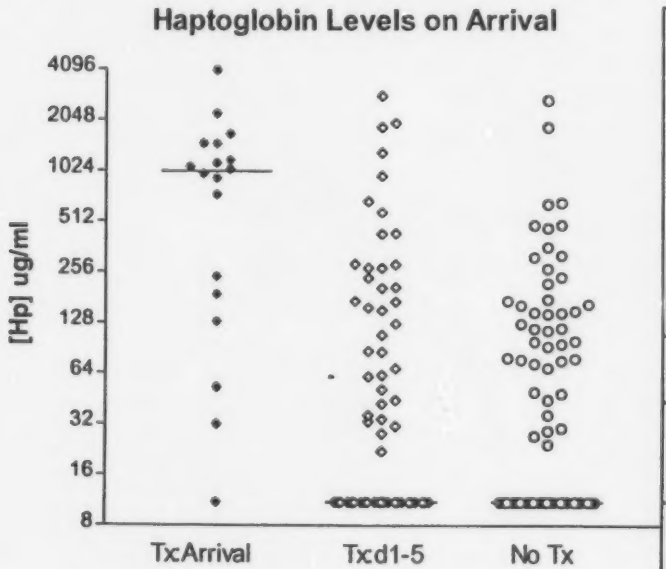


Figure 3.
Blood samples collected from calves at the time of entry to the feedlot were tested for serum haptoglobin concentration. The calves were classified as to whether they were treated with antibiotics on arrival (Tx:Arrival; n=18), or were pulled for treatment during the first 5 days in the feedlot (Tx:d1-5; n=82), or were not treated during the first 5 days in the feedlot (No Tx; n=144) The results are depicted as individual values, and the median haptoglobin concentration for each group. For the Day 1-5 Tx and No Tx groups the median value was below the detection limit of the haptoglobin assay (20 ug/ml).

The first group of calves had a temperature >=40.5 C and were treated with antibiotics on arrival (n=18). The second group of calves were pulled for treatment during the first 5 days in the

feedlot (n=82), and the third group were not treated during the first 5 days in the feedlot (n=144). Haptoglobin concentration was significantly higher ($p<0.001$) in the calves that had a fever on arrival, although there was considerable overlap in the range of haptoglobin detected in all groups. The haptoglobin levels in calves that were treated on days 1 to 5 in the feedlot were not different from untreated calves.

Of the 18 calves with fever that were treated on arrival, 13 had to be treated a second time in the initial 5 day period (Fig 4). Calves that recovered had a higher median haptoglobin level than calves that needed retreatment ($p<0.05$).

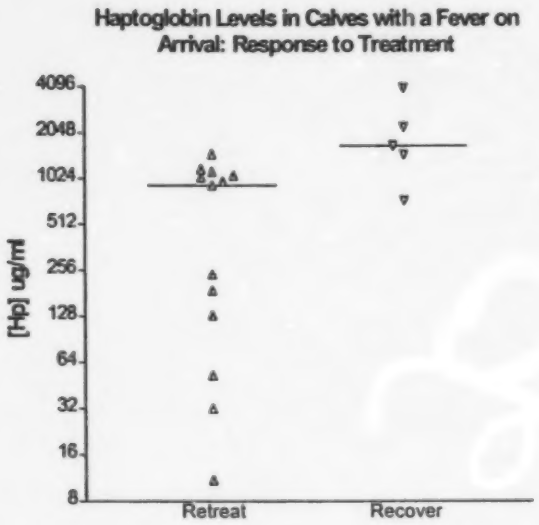


Figure 4.
Blood samples collected from calves at the time of entry to the feedlot were tested for haptoglobin concentration. Calves which had a temperature >=40.5 C and were treated with antibiotics on arrival (Tx:Arrival; n=18), were further classified as to whether they required retreatment during the first 5 days in the feedlot (n=13) or whether they recovered (n=5) The results are depicted as individual values, and the median haptoglobin concentration for each group. There is a significant ($p<0.05$) difference between retreated and recovered groups.

To determine if a threshold level of haptoglobin had diagnostic relevance, we used a concentration of >900 ug/ml to classify calves as having a high haptoglobin concentration. Of the 100 calves that were treated on arrival or in the first 5 days in the feedlot, 16% had high haptoglobin. This could be further broken down to 61% of calves with fever had high haptoglobin, while 6% of calves not treated on arrival, but subsequently pulled for treatment in the next 5 days had high haptoglobin. Only 1.4% of untreated calves had high haptoglobin.

Table 3. Classification of calves by clinical outcome and haptoglobin level.

	High Haptoglobin	
Description of calves	Number	Percentage
calves treated from arrival to day 5	16/100	16%
calves with fever	11/18	61%
calves treated on d1-d5	5/82	6%
calves with no treatment	2/144	1%
calves retreated after tx on arrival	7/13	39%
calves which recovered after intial tx	4/5	80%

Increased haptoglobin was associated with fever on arrival, but not with disease over the first 5 days in the feedlot. The results may have been biased since all calves received antibiotics on

arrival. We speculated that calves which recovered after a single treatment had a higher haptoglobin level because they were suffering from bacterial disease. Conversely, there were lower haptoglobin levels in calves that did not respond to antibiotic treatment because they had viral infections.

Use of Serum Haptoglobin on Entry to the Feedlot to Detect Disease

The objective of this study was to determine if a test for haptoglobin could discriminate between healthy calves and calves selected for treatment on entry to the feedlot. As well, we wished to determine if the level of haptoglobin could be used to predict the occurrence of disease during the first five days in the feedlot.

Blood samples were collected from calves at the time of entry to a commercial feedlot from Oct 15 to Dec. 1, 1997. Calves had their temperature and weight recorded at entry and 5 days post entry. Calves were only treated with antibiotics if their temperature was 40 C or greater or if they were considered sick by feedlot staff. Serum samples were stored at -20 C until analyzed for haptoglobin concentration by ELISA.

A total of 1333 animals were included in the study. They arrived in the feedlot in 19 different lots on 6 different dates. The study included 64 bulls, 397 heifers, and 872 steers. Upon arrival, 4.4% of the calves had a rectal temperature of 40 C or greater, and 20% of calves had detectable levels of serum haptoglobin. Of the 261 calves with detectable serum haptoglobin, 46 had a serum haptoglobin concentration greater than 800 ug/ml.

There was a significant association ($p=0.001$) between serum haptoglobin concentration and the presence of a fever on arrival. Haptoglobin was detected in 37% of calves with a fever, but only 18% of calves without a fever (Table 4). Similarly, there was a significant association between haptoglobin concentration and treatment on arrival ($p=0.001$). Eighteen percent of animals with undetectable haptoglobin were treated, while 28% of animals with elevated haptoglobin were treated. There was also a significant association ($p=0.001$) between gender of the animals and the serum haptoglobin concentration. Twelve percent of bulls had haptoglobin levels in excess of 800 ug/ml while only about 3% of steers and heifers had this level of haptoglobin (Table 4).

The objective of this study was to determine if the haptoglobin level on arrival was predictive of the occurrence of disease in the first five days in the feedlot. Of the 1333 animals in the trial, 1199 had their rectal temperature recorded during days 4, 5 or 6. The distribution of haptoglobin concentration and gender were similar in this subset to the total population. Of these animals, 48% had a fever and 65% were treated with antibiotics. There was no significant association between serum haptoglobin concentration and the occurrence of a fever ($p=0.22$) or the decision to treat with antibiotics ($p=0.67$).

Since a number of animals were treated with antibiotics on arrival and this may have confounded the relationship between haptoglobin and subsequent disease, the data was reexamined using only calves that were not treated on arrival. Again, there was no significant relationship between haptoglobin concentration and the subsequent occurrence of fever or treatment.

Table 4. Haptoglobin level in various categories of calves.

Descriptor	Category	Haptoglobin level[a]		
		< 50	50 to 799	>800
Fever at entry	Temp 1< 40 C	1035 (81.2%)	199 (15.6%)	40 (3.1%)
	Temp 1 >= 40 C	37 (62.7%)	16 (27.1%)	6 (10.2%)
Treatment at entry	No	875 (82.5%)	152 (14.3%)	34 (3.2%)
	Yes	197 (72.4%)	63 (23.2%)	12 (4.4%)
Gender	Bull	42 (65.6%)	14 (21.9%)	8 (12.5%)
	Heifer	323 (81.4%)	64 (16.1%)	10 (2.5%)
	Steer	707 (81.1%)	137 (15.7%)	28 (3.2%)
TOTAL		1072 (80.4%)	215 (16.1%)	46 (3.5%)

[a] *Calves were grouped into low, medium and high levels of serum haptoglobin concentration. The number of calves in each haptoglobin level and the percentage within the descriptor category are shown.*

Haptoglobin concentration was associated with fever on arrival, but was not predictive for disease (fever or treatment) in the next 5 days, even in calves that did not receive prophylactic antibiotics.

Use of Serum Haptoglobin Concentration to Discriminate Between Viral and Bacterial Disease and to Predict Outcome in Feedlot Calves with Bovine Respiratory Disease

The objectives of this study were to determine:

1) Is high haptoglobin concentration associated with bacterial but not viral disease? The hypotheses were:
   - calves with high concentrations of haptoglobin (>1000 ug/ml) would have a higher frequency of positive bacterial cultures from the respiratory tract than would calves with low haptoglobin levels.
   - with the assumption that bacterial infections would be more responsive to antibiotic therapy, calves with high concentrations of haptoglobin (>1000 ug/ml) would have a lower day 4 temperature in treated versus untreated calves. Calves with low haptoglobin should show no difference in temperature between treatment groups.
2) Is haptoglobin concentration predictive of disease outcome in non-treated calves? Disease outcome was characterized as fever at day 4, recovery, relapse, or death.

125 recently weaned, auction market derived, crossbred, beef calves in a commercial feedlot were selected as suffering from BRD on the basis of: rectal temperature >= 40.5°C on two consecutive days, and absence of clinical signs referable to organ systems other than respiratory system. The calves were allocated to two treatment groups, antibiotic treatment (n=84) and untreated controls (n=41).

A blood sample was collected and transtracheal wash and nasal swab obtained for culture on Day 0 (second day with elevated temperature). Rectal temperature was recorded on Days -1, 0, 1, 2, 3, 4. A sickness rank was assigned to each calf based on whether the calf died, relapsed twice or once, or recovered, and the day on which death or retreatment occurred. Serum samples were stored at -20 C until analyzed for haptoglobin concentration using a capture ELISA.

To determine if high haptoglobin concentration was associated with bacterial infections, we looked at the frequency of bacterial isolation from transtracheal washes and nasal swabs. Calves with high concentrations of haptoglobin (>1000 ug/ml) had a significantly ($p<0.05$ Fishers exact test) higher frequency of positive bacterial cultures from the respiratory tract than calves with low haptoglobin levels (Table5).

Table 5.

	Bacterial Culture Results[a]	
Haptoglobin[b]	Positive	Negative
High	36 (53%)	20 (35%)
Low	32 (47%)	37 (65%)

[a] *Transtracheal washes, nasal swabs and blood samples were collected on day 0. Isolation of P. haemolytica, P. multocida or H. somnus in any of these samples was recorded as a positive culture.*

[b] *Serum haptoglobin concentration on Day 0 was used to classify the calves into high (> 1000 ug/ml) and low groups.*

As another indicator of bacterial infection, we looked at the response rate to antibiotic therapy. In calves with high concentrations of haptoglobin (>1000 ug/ml), 29% of untreated and 3% of treated calves still had a fever on day 4 (Fig 5). In calves with low haptoglobin levels, an identical 29% of untreated calves and 2% of treated calves had a fever on day 4. Thus, responsiveness to antibiotics was not a distinguishing characteristic of high and low haptoglobin groups.

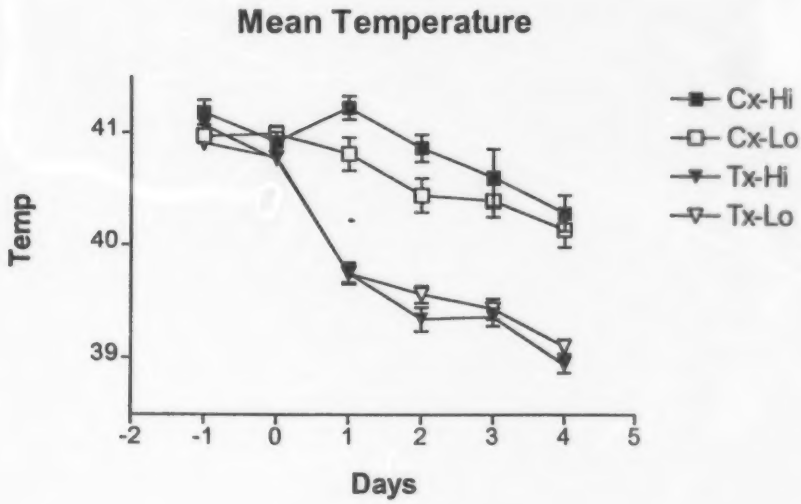


Figure 5.
Calves were grouped according to treatment group and Day 0 haptoglobin level (High haptoglobin >1000 ug/ml). Rectal temperature was recorded daily and the mean (SEM) for each group is depicted.

Next, the untreated calves were used to determine if haptoglobin concentration on Day 0 could be used to predict outcome. There was a significant correlation between haptoglobin concentration and sickness rank (Fig 6).

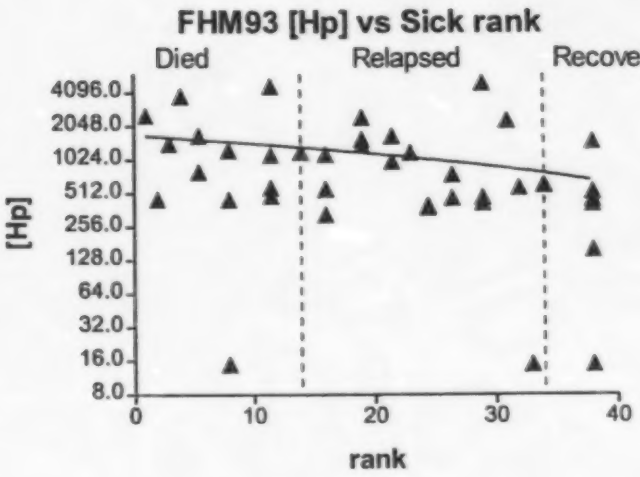


Figure 6.
Untreated calves (n=41) were assigned a sickness rank based first on outcome category and second by time of death or relapse. Sickness rank was significantly correlated with Day 0 haptoglobin concentration.

As well, if calves were classified by need for antibiotic treatment, i.e., calves which recovered didn't need treatment and calves which relapsed or died were grouped as requiring treatment, these groups had significantly different levels of haptoglobin ($p<0.05$) (Fig 7).

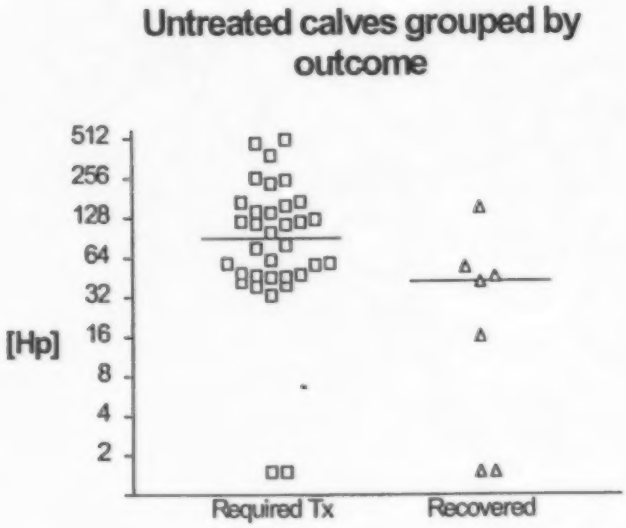


Figure 7.
Untreated calves (n=41) were classified by outcome as recovered (n=6) or required treatment (n=35;died or relapsed). The haptoglobin level for each calf and the median for the group is depicted.

Despite these relationships between haptoglobin concentration and disease severity, and a numerically higher percentage of calves with high haptoglobin in the diseased outcomes, high haptoglobin values were not predictive of death, relapse, or persistence of fever at day 4 (chi square analysis) (Table 6).

Table 6. Classification of calves by haptoglobin level and outcome.

	Outcome[a]			
Haptoglobin[b]	Dead	Relapsed	Fever at Day 4	Recovered
High	7 (54%)	9 (43%)	8 (35%)	1 (14%)
Low	6 (45%)	12 (57%)	15 (65%)	6 (86%)

[a] *Calves were classified as to whether they recovered uneventfully, relapsed or died. These categories are mutually exclusive. Calves were also classified as to whether their rectal temperature was >= 40.5 on Day 4.*

[b] *Serum haptoglobin concentration on Day 0 was used to classify the calves into high (> 1000 ug/ml) and low groups.*

In experimental models of BRD, we had observed that the haptoglobin response subsequent to viral (BHV-1) infection was not as strong as that following bacterial (P. haemolytica) infection. Thus, we speculated that the haptoglobin level may be able to distinguish between viral and bacterial disease. To test this hypothesis, we retrospectively analyzed the serum haptoglobin concentration of calves which were used to study antibiotic therapy of BRD in the feedlot. Calves with a high haptoglobin concentration did have an increased frequency of bacterial isolation from transtracheal washes or nasal swabs. However, our attempt to distinguish bacterial from viral disease by responsiveness to antibiotic therapy did not work in this study since virtually all of the calves responded to antibiotic therapy. Thus, our assumption that a proportion of calves selected for treatment on the basis of elevated temperature would be suffering from a viral infection only was false. That high haptoglobin was associated with bacterial isolation then may not be due to a discrimination between viral and bacterial disease, but an association of high haptoglobin with a more fulminant infection such that bacteria were of sufficient number to be more readily isolated.

When we examined the ability of the haptoglobin concentration on Day 0 to predict outcome in the untreated calves, we were able to demonstrate a relationship between haptoglobin concentration and sickness rank. As well, calves that recovered uneventfully had significantly lower haptoglobin levels than calves which relapsed or died. However, the relationship between haptoglobin concentration and outcome was not so strong that haptoglobin levels could be used to clearly discriminate between the different outcomes, death, relapse or recovery, or predict the outcome of individual calves.

H. somnus experimental challenge

Since the results obtained in our feedlot studies did not demonstrate a clear diagnostic use for haptoglobin testing, the objective of this study was to determine if haptoglobin levels in cattle infected with *Haemophilus somnus* are elevated, since *H. somnus* is an important cause of disease in the feedlot.

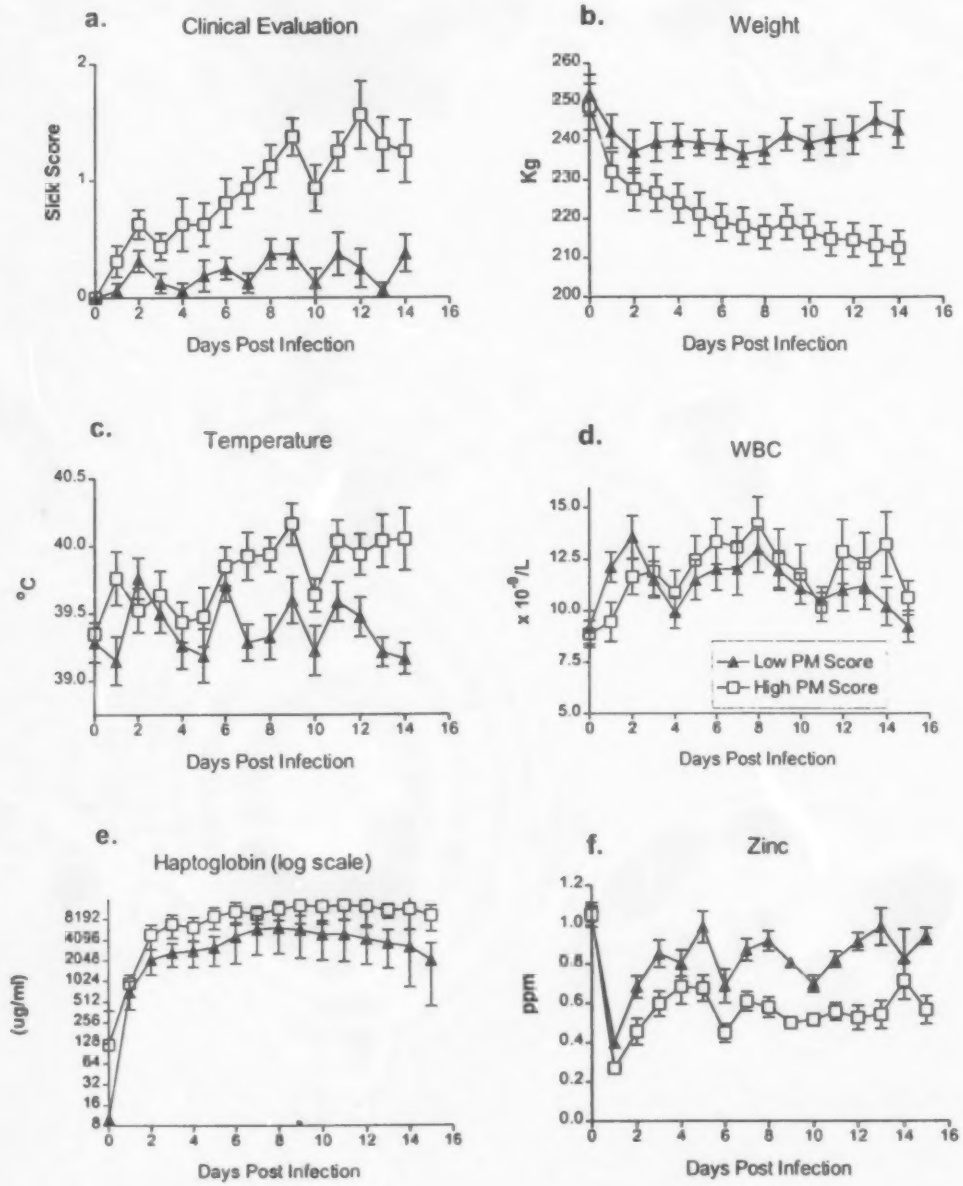
Sixteen calves were administered *H. somnus* by intravenous injection. The calves were examined daily for signs of clinical disease, their temperature and weight were recorded, and blood samples were drawn for CBC, serum haptoglobin, and plasma zinc analyses. At 15 days post infection, all calves were killed and examined for postmortem lesions. On the basis of the postmortem evaluation, the calves could be divided into two groups, those with high and those with low cumulative postmortem scores.

Using this grouping of the calves, the results were analyzed to determine which of the measured parameters best reflected the severity of disease (Fig.8). Calves with more severe lesions (High PM score group) had more severe clinical signs. Calves with more severe lesions had greater impairment of performance, as measured by weight gain. Thus, the use of postmortem scores to group the calves reflected the severity of disease. However, the temperature of the calves rose only slightly in the first two days after infection, and did not separate the groups until after day 6. White blood cell counts, often used to monitor infection, rose slightly in both groups initially, but could not be used to distinguish between the groups. Thus, these commonly used tests were poor at detecting the onset of infection and reflecting its severity.

Two parameters of the acute phase response were also monitored. Haptoglobin levels began to rise in all animals by 1 day after infection and rose to extremely high levels in the high PM score group (exceeding 10,000 μg/ml on day 6). The low PM score group had lower, but still very elevated haptoglobin levels, with a maximum mean value of about 6000 μg/ml on day 8, and finished the trial at about 2000 μg/ml, which is still much above the normal healthy value of <100 μg/ml. Thus, both groups showed a response to infection, measurable by 24 h after infection, and the severity of the disease was reflected in the magnitude of the response.
Zinc levels dropped precipitously on day 1 and then slowly rose over the next three or four days. The low PM group was characterized by a more rapid rise and return to normal values. The high PM group never did return to baseline levels.

These results again illustrate the benefit of monitoring the acute phase response to both detect disease and characterize its severity. *H. somnus* infection appears to be a particularly potent activator of the haptoglobin response. Measurement of zinc appears to be particularly well suited to the detection of disease very soon after onset. However, the transient nature of the maximal zinc response may limit its practicality at this time, as it would only be effective if used soon after infection.

Figure 8. Mean daily measurements for calves infected with H. somnus and grouped by postmortem score.



Reaction to Injections and Other Management Practices

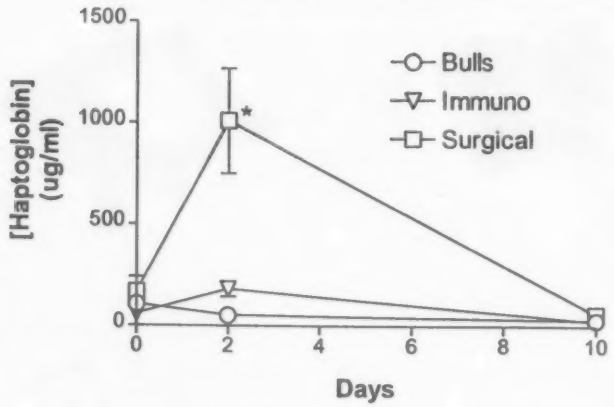
As well as being used to detect disease, monitoring the acute phase response can give an objective assessment of the degree of tissue injury associated with various procedures. The haptoglobin response was evaluated to the following:

- Castration
- Antibiotic injection

- Vaccination

In collaboration with Drs. Van Kessel, Stookey, and MacKinnon of the Animal Science Dept. and WCVM, we analyzed serum samples of calves following immunologic and surgical castration. Sixty calves were assigned to three groups. One group was immunized to cause "castration" by interference with the hormonal control of reproduction, one group was surgically castrated, and the third group was left as bulls. Serum samples were obtained just prior to booster immunization and surgery, and at two and 10 days after the procedures. Although there were a few animals in each group with elevated levels of haptoglobin at the start of the experiment, there was a significant increase in mean haptoglobin concentration in the surgical group on day 2 (Fig 9).

Figure 9. **Effect of Castration on Haptoglobin Levels**



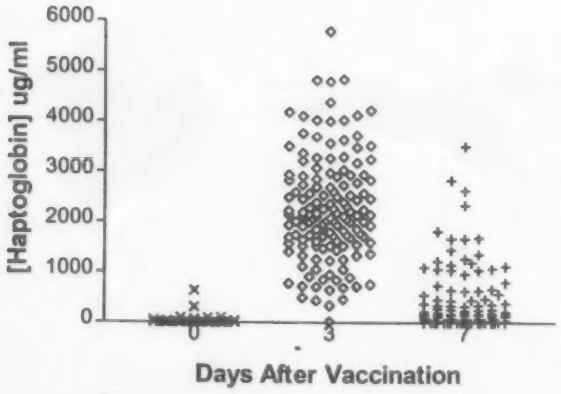


Figure 10. Haptoglobin response after vaccination.

Haptoglobin Response Following Vaccination

We also monitored the reaction to vaccination with *P. haemolytica* and Clostridial vaccines at 3 and 7 days post immunization in 140 weaned beef calves (Fig. 10). Prior to vaccination, 99% of the calves had a haptoglobin concentration less than 500 μg/ml. At day 3, only 4% of the calves

had haptoglobin below this level, but by 7 days after vaccination, 78% of calves were back under 500 µg/ml. That a haptoglobin response of this magnitude was induced following a procedure routinely applied to calves entering the feedlot has implications for the use of haptoglobin testing in the feedlot.

b) Develop Other Tests To Monitor The Acute Phase Response

We believe that assessment of an animal's health status can be improved by monitoring more than one acute phase reactant. Interleukin-6 was targeted as another candidate for acute phase reaction monitoring because of its pivotal role in regulating the acute phase response. When the project was initiated, the gene for bovine IL-6 had been isolated and cloned at VIDO. Our objectives were to:

1. express recombinant bovine IL6 protein using this gene
2. produce polyclonal and monoclonal antibodies to bovine IL6
3. use these reagents to develop an immunoassay that would measure the level of IL6 in blood.

Although simple in concept, this aspect of the project suffered from technical problems, and progress was not made as quickly as originally anticipated. The results achieved include: Recombinant bovine IL6 protein was expressed using multiple strategies, and used to produce polyclonal and monoclonal antibodies.

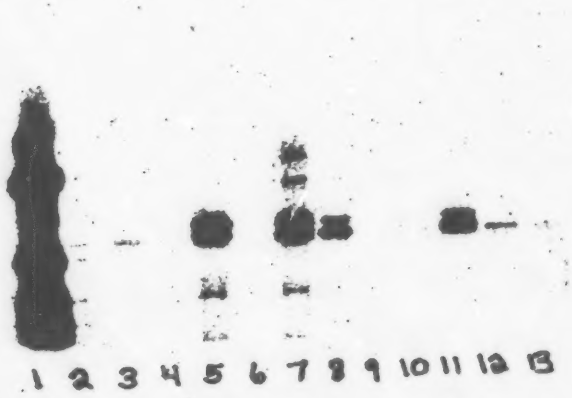


Figure 11
The gene for bovine IL-6 was expressed in an in vitro transcription-translation system (Promega IVTT®). The resulting IL6 gene product was radiolabelled with ^{3}H-leucine. This product was incubated with serum from a rabbit and a mouse immunized with gD:IL6 and culture supernatants from putative monoclonal antibody producing hybridomas. The antibody and bound IL6 was removed from the reaction mixture by immunoprecipitation with Protein G Sepharose. The amount of bound IL6 was determined by B-spectroscopy. Lane 1: Mol. Wt. Markers; 2. Buffer; 3. Culture media; 4. Mouse serum before immunization; 5. -after immunization; 6. Rabbit serum before immunization; 7 - after immunization; 8. Clone 241; 9. Clone 261; 10. Clone 421; 11. Clone 47-8; 12. Clone 499-18; 13. Clone 499-24.

Antibodies from both the rabbit and mouse immunized with gD:IL6 and three hybridoma clones recognized the labelled IL6 (Fig 11).

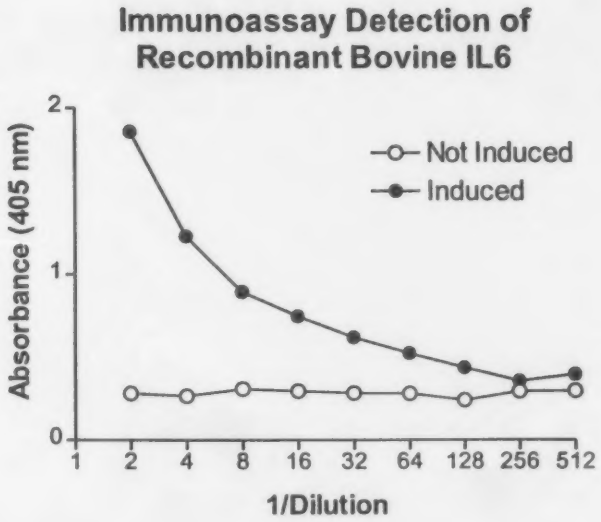
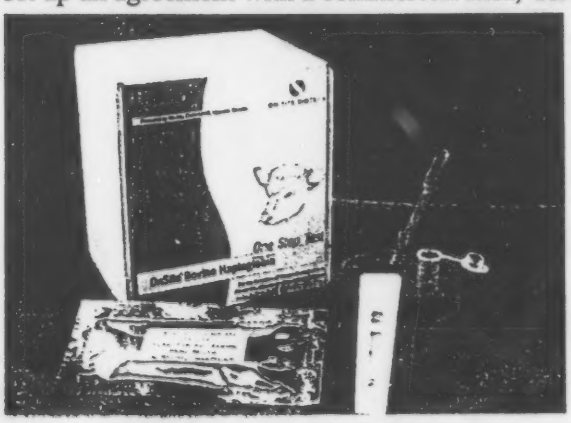


Figure 12
ELISA plates were coated with rabbit anti-BoIL6 and then incubated with supernatants from E. coli containing the Trx:IL6 gene. In one culture the E.coli were treated to induce production of the IL6 fusion protein. Any IL6 captured by the rabbit antibody was detected by addition of monoclonal antibody 47, and appropriate color development reagents. The antibodies reacted only with the induced supernatant and in a dilution dependent manner.

Using the reagents produced to date, an ELISA was developed which could detect recombinant bovine IL6 (Fig 12). However, using this assay, no IL6 was detected in serum samples from cattle experimentally infected with *H. somnus*. It is likely that the test will have to be modified to increase its sensitivity before it will be effective in detecting IL6 in blood samples. Unfortunately, the project reached its conclusion before any more developmental work could be done on the assay.

c) Improve Test Formats To Make Them More Practical For Field Use

To develop an assay format for haptoglobin which would be more practical for field use, we set up an agreement with a commercial firm, On-Site Biotech, Uppsula, Sweden for the development of a rapid diagnostic kit for haptoglobin. This firm has expertise in the area of rapid diagnostic tests, and with the antibody and purified haptoglobin reagents we provided, they produced a prototype "cowside" test.. The kit was done up professionally and it worked with a limited number of serum samples. However, this test was too sensitive for practical application (sensitivity of about 30 µg/ml). That is, it could detect levels of haptoglobin which were not necessarily associated with significant disease. Healthy cattle have a haptoglobin concentration below 30 µg/ml, but haptoglobin can become elevated above this level due to relatively minor insults such as vaccines or minor surgery such as castration. Therefore, we asked the company to modify the test to give a cutoff of 500 ug/ml. This level of sensitivity was based on results from an experimental model of bovine respiratory disease (BRD) (aerosol challenge with bovine herpesvirus-1 and *Pasteurella haemolytica*), and the field



study of feedlot cattle treated for BRD. Using a cutoff of 500 ug/ml, we maintain a sensitivity of 85% or better.

Serum samples, which had been tested twice by ELISA, were selected from three different trials, a feedlot trial, a slaughterhouse trial, and a weaning experiment with 6-month-old calves. Using a positive-negative outcome for the Onsite test, there were a fair number of false positives and false negatives compared to a cutoff of either 500 or 100 ug/ml ELISA concentration (Table 7).

Table 7. Comparison of On-Site Test with haptoglobin ELISA results.

	On-Site Test	
ELISA	negative	positive
<500	13	20
>500	4	24
<100	5	5
>100	12	39

While modification of the test is required to improve its performance, the biggest problem is defining the situation and the cutoff level at which haptoglobin testing is diagnostic.

3.9.4 Conclusions:

Over 2500 serum samples were tested for haptoglobin concentration in the four feedlot studies conducted. In all studies there was a significant association between haptoglobin and disease. However, in all studies, there was not a clear discrimination between sick and healthy calves based on haptoglobin. Recently published studies on haptoglobin testing in feedlot cattle came to similar conclusions. In some cases, this lack of discrimination could be due to a haptoglobin response to a noninfectious cause, since we also demonstrated increases in haptoglobin concentration following vaccination, antibiotic injection, and castration. Some discrepancy is probably also due to not having a definitive way of detecting disease, and having to rely on subjective assessment for categorizing diseased and healthy animals. As well, while increases in haptoglobin have been demonstrated in a number of diseases, little work has been done on the factors that cause haptoglobin levels to decline. In fact, we saw rapid declines in haptoglobin following antibiotic treatment. A better understanding of the downregulation of haptoglobin levels may increase our ability to utilize haptoglobin concentration as a diagnostic aid. Should that diagnostic niche be found, we have developed, in collaboration with OnSite Biotech, a rapid "cowside" test that would allow haptoglobin testing in the field. One application for haptoglobin testing may be in the research setting, where it can be used as an objective assessment of disease severity or tissue damage.

We had hoped to overcome some of the vagaries of haptoglobin testing by producing a test for another acute phase reactant, thus increasing our assessment of the acute phase response. Unfortunately, the development of the interleukin-6 assay did not proceed as expeditiously as planned. However, we are now at the stage that the required reagents are available to develop a test to measure serum IL6 levels and begin to investigate the association of IL6 and disease.

3.9.5 Information of Benefit to Producers:

The early detection of disease in livestock is a critical component of health management in order to in initiate effective treatment and reduce losses. Since the immune system of the animal has evolved to recognize and respond to foreign microorganisms, we can detect infections quickly by monitoring the immune response. In this project, we measured the levels of haptoglobin in the blood of cattle. Haptoglobin concentration rose dramatically soon after infection or injury. Thus, measurement of haptoglobin improved our ability to detect a variety of diseases, as well as our ability to monitor the response to treatment. This test could be of benefit on the farm, in the feedlot, or at the packing plant, since technologies exist to make this test rapid and portable. While haptoglobin measurement was not capable of detecting all disease situations, just as taking the temperature is not, it is another example of how monitoring the animal's response provides us with information to improve health management.

3.10 Project: Development of models for evaluation of mucosal immune responses

3.10.1 Objectives:

Approximately 90% of all pathogens that cause disease in domestic animals enter the body through the mucosal surfaces of the eyes, respiratory system, intestinal tract, and the reproductive tract. Prevention of these diseases will require vaccines that can induce protective immunity at these surfaces. The primary objective of this project was to develop animal models to assess the capacity of adenovirus vaccine vectors and other oral vaccine delivery systems to induce effective mucosal immunity.

3.10.2 Background and Approach:

For these investigations we used a variety of adenovirus vaccine vectors. The first vector was human adenovirus 5 vector which had the glycoprotein D (gD) gene of bovine herpesvirus-1 (BHV-1) inserted into the E3 region of the viral genome (HAd5-gD/E3. This vector was used to establish immunological assays to detect gD-specific antibody and cell-mediated immune responses in sheep and to evaluate ruminant animal models for testing adenovirus vaccine delivery. This work was then extended to bovine adenovirus vaccine vectors. Finally we developed an oral vaccine delivery technology, alginate microspheres, that could potentially be used to deliver viral vaccine vectors, protein antigens, or vaccine adjuvants.

A surgical procedure was also developed which permitted the preparation of sterile intestinal-loops in fetal lambs without disrupting normal intestinal function. These intestinal-loops either contained or lacked the lymphoid tissue thought to be necessary for the induction of mucosal immune responses. After the lambs were born it was then possible to inject vaccines directly into the surgically prepared intestinal-loops. This model allowed us to address questions regarding vaccine targeting and delivery and to quantify the induction of mucosal immune responses following vaccine delivery.

3.10.3 Results:

a) Establish immune assays.

Intradermal injection of human adenovirus vaccine vector (HAd5-gD/E3) into sheep induced both antibody and cell-mediated immune responses to the gD protein. This confirmed that the vaccine vector worked in the sheep model and allowed us to establish the necessary immunological assays to evaluate mucosal immune responses. Injection of the vector into surgically prepared intestinal-loops of 6-8 week old lambs demonstrated that this was an effective way to induce both mucosal and systemic immune responses. Furthermore, the injection of the HAd5-gD/E3 vector into intestinal-loops of newborn lambs confirmed that the mucosal immune system of the neonate was functional (Figures 1-3). In contrast, intradermal injection of the vector induced systemic immunity but no detectable mucosal immunity. Thus, enteric immunization with adenovirus vaccine vectors was an effective way to induce mucosal immunity. This was the first step in establishing the feasibility of using an adenovirus vaccine vector as an oral vaccine.

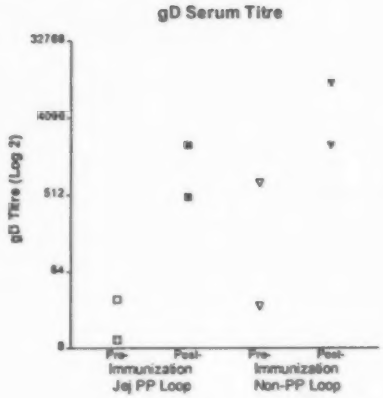


Figure 1. gD titre in serum after injection of 1 X 10^9 pfu of HAd5-gD/E3 into the intestine of 2-4 day-old lambs. Serum was collected pre-immunization and weekly for one month post-immunization. Maximal titres were observed 3 weeks post-immunization and these values are presented for individual lambs. The gD titres were determined with an ELISA that detected total gD-specific immunoglobulins.The adenovirus vector was injected into intestinal-loops that contained either a jejunal Peyer's patch (jej PP loop) or no Peyer's patch (Non-PP).

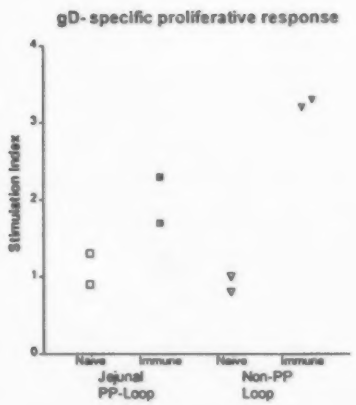


Figure 2 . gD-specific lymphocyte proliferation responses after injecting 1 X 10^9 pfu of Had5-gD/E3 into the intestine of 2-4 day-old lambs.
Lambs were killed four weeks after immunization and splenocytes were cultured for 72 h with 1 μγ/ml of purified gD protein or medium alone (Background). Cultures were pulsed with 20 μCi [H^3]-thymidine for 6 h before using routine cell harvesting methods to quantitate [H^3]-thymidine incorporation as counts per minute (cpm). Stimulation index was calculated by dividing the cpm with gD protein by the cpm in Background cultures. Four replicate cultures were counted for each lamb and data presented are mean values for individual lambs. Adenovirus vector was injected into intestinal-loops that contained either a jejunal Peyer's patch (jej PP loop) or no Peyer's patch (Non-PP).

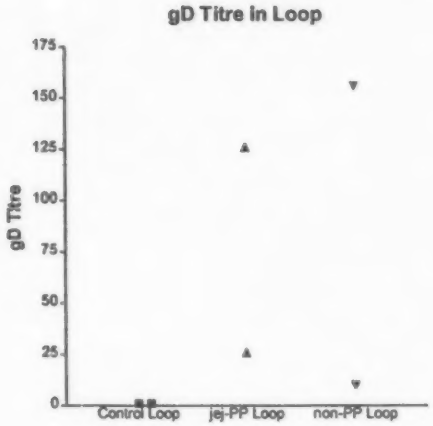


Figure 3. The gD titre in intestinal-loops following injection of 1 X 10^9 pfu of HAd5-gD/E3 into the gut loops of 2-4 day-old lambs. Intestinal-loops contents were collected when lambs were killed four weeks after immunization. The gD titres were determined using an ELISA that detected total gD-specific immunoglobulin. The adenovirus vector was injected into intestinal-loops that contained a jejunal Peyer's patch (jej PP loop), or no Peyer's patch (Non-PP loop). The background titre in the ELISA was determined by collecting the contents of intestinal-loops (Control loop) that were not injected with adenovirus vector.

b) Determine duration of immunity induced following enteric vaccination of neonates.

Human adenovirus vaccine vector, expressing the gD protein of bovine herpesvirus-1 (HAd5-gD/E3), was injected into either intestinal-loops (Group I) or subcutaneously (Group II) in 1-3 day old lambs. Immunological assays were used to measure systemic (blood) immune responses to confirm that the lambs had responded to the vaccine vector. The two groups of lambs were designed to determine if oral immunization was as good or better than subcutaneous vaccination for the induction of long-term immunity. Six months later the lambs were given a second immunization to determine if there was immune memory for the gD protein. The 6-month old lambs were boosted with gD protein formulated in liposomes and the vaccine was injected into the trachea. This route of immunization was chosen as a way to mimic a respiratory infection with BHV-1. Five weeks after the secondary immunization the lambs were euthanized and tissues were collected to analyze immune responses in the gut and lungs (mucosal immunity) and the spleen (systemic immunity).

The analysis of serum antibody, 6 months after neonatal immunization, revealed that oral immunization was less effective than intradermal immunization for the induction of a long-term serum antibody response (Figure 4). However, following a secondary intratracheal immunization it was evident that oral immunization was a more effective route for the induction of long-term immune memory for antigens delivered to a mucosal surface. This type of immune memory should provide better disease protection against viruses and bacteria that enter the lungs and cause pneumonia in weaned calves.

The analysis of local immune responses in the lung and gut provided further evidence that oral (gut-loop) immunization had induced long-term immune memory. The analysis of gut-associated lymphoid tissue (Peyer's patches-PP) revealed a remarkable persistence of gD-specific cells for 6 months after immunizing the newborn lamb. These antigen-specific cells were identified as antibody secreting cells (Figure 5) and antigen-specific T cells that proliferated and secreted interferon-gamma (IFNγ) in response to gD protein (data not shown). No gD specific immune cells were detected in the PP of lambs that were immunized intradermally as newborns (Figure 5). This

revealed the importance of vaccine delivery to mucosal surfaces for the induction of mucosal immunity.

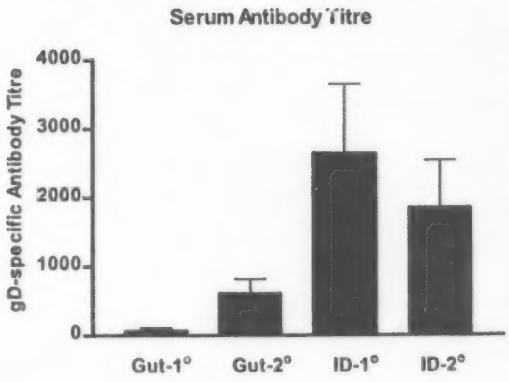


Figure 4. Newborn lambs were immunized with 1 X 10^9 infectious particles of HAd5-gD/E3 vaccine vector in gut-loops (Gut; n = 4) or intradermally (ID; n = 4). Six months later, the level of gD-specific serum antibody was assayed by ELISA. No detectable antibody was present in lambs immunized in the gut (Gut-1°) but antibody persisted for 6 months in lambs immunized intradermally (ID-1°). Serum antibody titres were then assayed 2 weeks after a secondary immunization by intratracheal injection of gD protein. This secondary immunization induced a significant increase in serum antibody in lambs previously immunized in the gut (Gut-2°) but not lambs previously immunized intradermally (ID-2°) with the HAd5-gD/E3 vaccine vector.

Another finding of considerable importance was the observation that a primary oral immunization (gut-loops) was able to transfer immune memory to the lung. This conclusion is based on the observation that gD-specific ASC were detected in the lung lymph nodes of lambs immunized as neonates in gut-loops as neonates (Gut-Lung) but no ASC were detected in the lung lymph nodes of lambs immunized intradermally (Figure 5). The presence of ASC is a good indication of local mucosal immunity. Antigen-specific T cells (IFNγ secretion and proliferative responses to gD) were detected in the lung lymph nodes and the spleen of both groups of lambs (data not shown). This confirmed that both intradermal and gut-loop immunization of newborn lambs had induced an immune memory that had spread throughout the body. Thus, oral immunization should be effective for the induction of long-term immune memory at both mucosal surfaces (gut and lung) and systemic organs (spleen).

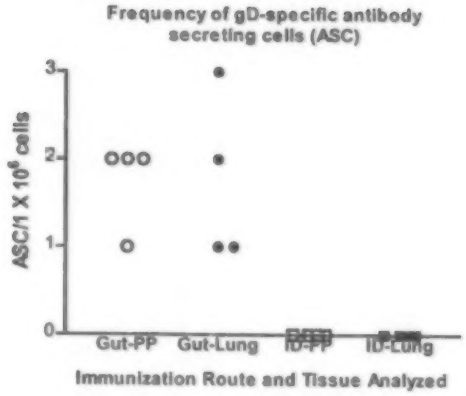


Figure 5. Newborn lambs were immunized with 1 X 10^9 infectious particles of HAd5-gD/E3 vaccine vector in gut-loops (Gut) or intradermally (ID). Six months later, the lambs were immunized a second time by injecting gD protein intratracheally. The frequency of gD-specific antibody secreting cells (ASC) was then assayed in the gut lymphoid tissue (PP) or tracheobronchial lymph node (Lung). The gD-specific ASC were present in both the gut (Gut-PP) and lungs (Gut-Lung) of lambs that were immunized in gut-loops when 1-3 days old. However, no ASC were detected in the gut (ID-PP) or lungs (ID-Lung) of lambs that were immunized intradermally when 1-3 days old.

c) Optimize adenovirus vaccine vector use in cattle.

Dr. Suresh Tikoo, working with scientists at VIDO, developed a bovine adenovirus vaccine vector (BAd3). The bovine herpesvirus-1 gD gene was inserted in the E3 region of this vector (BAd3-gD/E3) and a large quantity of the vaccine vector was produced in our laboratory. Experiments were completed with two groups of newborn lambs. The first group of lambs was used to determine if the BAd3-gD/E3 vector caused clinical disease in newborn lambs. This was done to establish the safety of a live virus vaccine vector in newborn ruminants. The second group of lambs was used to compare immune responses induced by different doses of the BAd3-gD/E3. This information was necessary to determine if the information generated with the human adenovirus vector could be extrapolated to the bovine adenovirus vector. This would provide a basis for directly applying the dose and route of administration from the sheep model to the immunization of newborn calves with the bovine adenovirus vector.

To examine the safety of the bovine adenovirus vector three groups of newborn lambs were orally immunized with different doses of the BAd3-gD/E3 vector. All lambs were colostrum-deprived to ensure there was no passive immunity against the vaccine vector. The first group of lambs was challenged with 2 X 10^{10} infectious units of BAd3-gD/E3 vector, the second group received 2 X 10^9 infectious units of BAd3-gD/E3 vector, and the third group received 2 X 10^8 infectious units of BAd3-gD/E3. All lambs were monitored for three weeks for signs of clinical disease (appetite, body temperature, respiration, diarrhea or abdominal pain) and fecal samples were collected to assay vaccine vector shedding. Lambs in all groups remained clinically normal and no vaccine vector was shed in the feces. These results indicated that the bovine adenovirus vector was non-pathogenic in newborn ruminants.

The second experiment evaluated the dose of BAd3-gD/E3 vector that induced an immune response in newborn lambs to determine if the immunogenicity of the BAd3 vector was equivalent to the human adenovirus vector. Two groups of three lambs were immunized by subcutaneous injection of either 2 X 10^{10} infectious units (Group I) or 2 X 10^8 (Group II) infectious units of BAD3-gD/E3 vaccine vector. Four weeks later the lambs were euthanized and tissues were collected to assay immune responses in various organs.

The analysis of serum antibodies revealed that the BAd-gD/E3 vector was immunogenic and induced a specific response to the gD protein (Fig. 6). However, this response was dose-dependent and only two Group II lambs displayed a low response. Further analyses of the immune responses in various lymphoid tissues revealed a much different picture. The analysis of gD-specific lymphocyte proliferative responses (Fig. 7) and IFNγ secreting cell frequency (data not shown) revealed that an equivalent number of gD-specific T cells were present in the lymph nodes and spleens of lambs in both groups. This confirmed that even the lower dose of BAd3-gD/E3 vector was immunogenic. A specific immune response was also detected in the gut-associated lymphoid tissue (jejunal Peyer's patches) of lambs in group I. This observation is quite remarkable since subcutaneous injection of the human adenovirus vaccine vector (Had5-gD/E3) never induced antigen-specific cells in gut-associated lymphoid tissue. This observation raises the exciting possibility that the bovine adenovirus vaccine vector can be delivered either orally or by subcutaneous injection to induce both systemic and mucosal immunity.

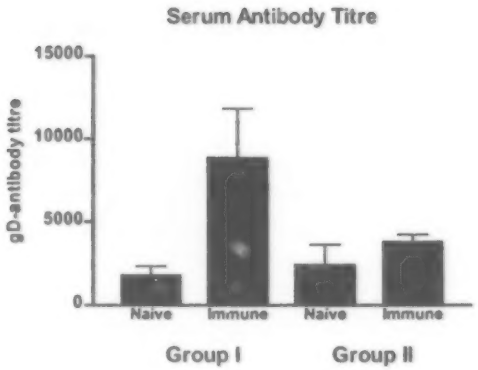


Figure 6. Newborn lambs were immunized subcutaneously with 2 X 10^{10} infectious particles (Group I; n = 3) or 2 X 10^{8} infectious particles (Group II; n =3) of BAd3-gD/E3 vaccine vector. Serum antibody titre for gD protein was assayed before (Naïve) and 4 weeks after immunization (Immune). Lambs immunized with 2 X 10^{10} infectious particles displayed a strong serum antibody response to the gD protein.

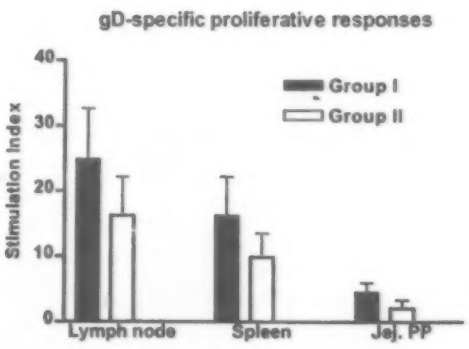


Figure 7. Newborn lambs were immunized subcutaneously with 2 X 10^{10} infectious particles (Group I; n = 3) or 2 X 10^{8} infectious particles (Group II; n =3) of BAd3-gD/E3 vaccine vector. The gD-specific proliferative responses were assayed for cells isolated from different tissues. An equivalent immune response was seen for both groups of lambs when analyzing cells from the draining lymph node (lymph node) and the spleen. There was a significant response in the gut-associated lymphoid tissue (Jej. PP) of Group I lambs. Only one Group II lamb displayed a significant proliferative response when cells from the intestine (Jej. PP) were assayed

d) Optimal dose and route of immunization for bovine adenovirus vaccine vector.

Investigations with the Human Adenovirus 5 vector established that oral immunization of newborn ruminants was feasible and induced both a mucosal and systemic immune response. The next step in these investigations was to establish that the Bovine Adenovirus 3 (BAD) vector and the Human Adenovirus vectors were equally immunogenic. This would ensure that results obtained with the Human Adenovirus vector could be extrapolated to the BAD vector. The immunogenicity of the BAD vector was evaluated by comparing the immune responses induced by two different doses of the BAD vector. Two groups of 5 lambs were immunized with either 1 X 10^8 or 1 X 10^{10} plague forming units (pfu) of purified Bovine Adenovirus 3 vector (BAD3) with the gD gene of bovine herpesvirus-1 inserted in the E3 region (gD/E3). In previous experiments, lambs were immunized with 1 X 10^9 pfu of the Human Adenovirus vector. This experiment confirmed that the BAD-gD/E3 vector was immunogenic in the lamb model with induction of a strong gD-specific immune response (Fig. 8). Furthermore, it appeared that a lower dose of the BAD vector (1 X 10^8 pfu) could be used than had been used with the Human Adenovirus vector (1 X 10^9 pfu).

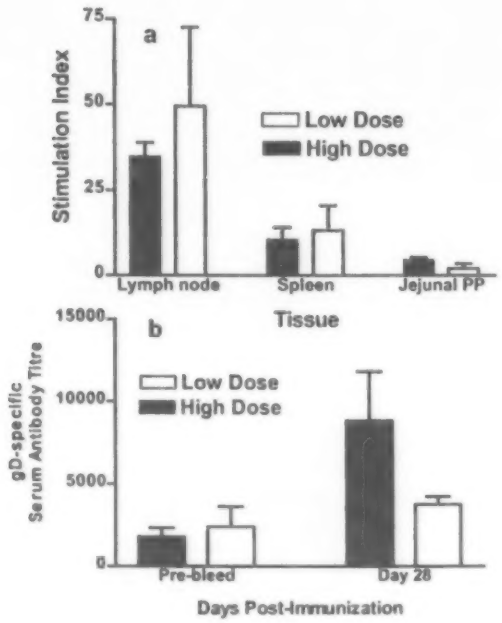


Figure 8

(a) Proliferative responses to the gD protein were assayed 4 weeks after lambs were given a single subcutaneous injection of 1 X 10^8 (Low Dose) or 1 X 10^{10} (High Dose) pfu of BAD3-gD/E3.). Responses were observed in the lymph node draining the injection site, in the spleen, and in the jejunal Peyer's patches (Jejunal PP). Jejunal PP responses were detected only in lambs injected with the High Dose.

(b) Serum antibody levels were measured 4 weeks after lambs were given single subcutaneous injection of 1 X 10^8 (Low Dose) or 1 X 10^{10} (High Dose) pfu of BAD3-gD/E3.). The High Dose induced a significantly greater level of gD-specific serum antibodies.

Mucosal immunity is the most effective form of immune protection against diseases that infect the lungs (i.e. pneumonia) and the intestine (i.e. diarrhea). However, vaccine induction of mucosal immunity requires vaccine delivery to the mucosal-associated lymphoid tissue. The present experiments were designed to identify the optimal delivery route to ensure that the BAD vaccine vector induced strong mucosal immunity. Four groups of lambs were immunized twice with BAD vaccine vector and the immunizations were performed either orally or subcutaneously (Table I). Systemic and mucosal immune responses were assayed 10 days after the second immunization.

Table 1. Oral and subcutaneous immunization with the Bovine Adenovirus vector.

GROUP[1]	Primary Immunization[2]	Secondary Immunization[3]
I	ORAL[4]	ORAL
II	ORAL	SQ[5]
III	SQ	SQ
IV	SQ	ORAL

1. n = 4
2. Lambs were 1-4 days old when immmunized with 1 X 10^9 pfu BAD3-gD/E3
3. Lambs were 3 weeks old when re-immmunized with 1 X 10^9 pfu BAD3-gD/E3
4. Vaccine was injected into the duodenum
5. Vaccine was injected subcutaneously (SQ) on the neck. The prescapular lymph node drains this injection site.

These experiments demonstrated that the route of BAD vaccine vector delivery had a significant impact on the induction of both systemic and mucosal immune responses (Fig. 9). It was evident that a combination of oral and SQ immunization induced the strongest mucosal immune responses. This was evident when analyzing local antibody production (Fig. 9b and c) and when analyzing cell-mediated immune responses (data not shown). On the basis of these results we are now designing experiments to evaluate the immune protection induced by BAD3 vaccine vector following immunization of newborn calves.

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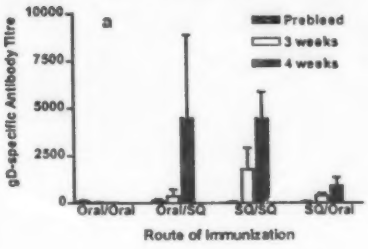
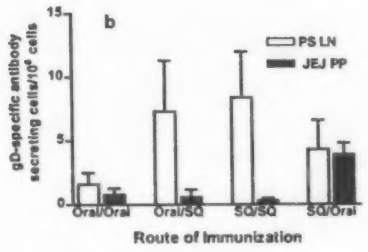
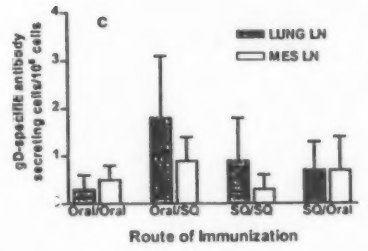
Figure 9. Route of BAD vector delivery alters systemic and mucosal immune responses.



(a) Analysis of gD-specific antibody in serum revealed that the strongest systemic immune response was observed following either an oral/SQ or SQ/SQ immunization. This confirmed that oral immunization primed the immune system and, as expected, the greatest antibody response was seen following SQ immunization.



(b) The systemic (prescapular lymph node: PS LN) and intestinal (jejunal Peyer's patch: JEJ PP production of antibody was assessed. Oral immunization did not induce systemic immunity but did induce mucosal immunity, especially when oral immunization followed a SQ priming. As expected, SQ immunization induced a strong systemic immune response but no mucosal immune response.



(c) Mucosal antibody production was further evaluated with cells isolated from lymph nodes draining the intestine and the lung. Mucosal immune responses were detected in both the lung and the intestine and the responses were strongest following oral/SQ immunization.

e) Develop an oral delivery system for the bovine adenovirus vaccine vector.

Previous work established that the gut-associated lymphoid tissue of newborn lambs could respond well to the adenovirus vaccine vector and that maternal antibody does not interfere with this immune response. Furthermore, we established that immunization in the intestine also induced immunity in the lung. This suggested that oral immunization could be used to prevent both respiratory and enteric infections. Therefore, work was initiated to develop a practical oral vaccine delivery system for newborn calves.

An ultrasonic nozzle (Turbosonic Inc., Waterloo, ONT.) and a Syringe Pump system (Harvard Apparatus, St. Laurant, Qûe.) were purchased in June, 1999. This system was assembled with a nitrogen gas injection system that permitted the aerosolization of liquids, through the ultrasonic nozzle, using controlled flow rates at constant pressures. This system was then used to evaluate several parameters to identify the optimum conditions required to consistently produce stable microspheres that were less than 10 μm diameter.

Table 2. Optimal conditions for the production of alginate microspheres.

Parameter Evaluated	Tested Range of Variation	Optimal Conditions
Alginate Viscosity	Low, Medium, High	**Medium**
Percent Alginate	0.5 to 2%	**1%**
Infusion Pressure	10 to 40 p.s.i.	**40 p.s.i.**
Infusion Rate	2 to 20 ml/minute	**2-5 ml/minute**
Percent Calcium Chloride	1.5 to 2.0 %	**%**
Aerosolization Distance	20-50 cm	**35 cm**

The six parameters that were systematically evaluated to identify the optimal conditions required to produce microspheres are summarized in Table I. The range of values tested for each paramter are indicated. Optimal production conditions were identified those that produced microspheres that were consistently over 80% less than 7-10 μm in diameter. This size restriction was considered essential since the gut-associated lymphoid tissue (Peyer's patches) can efficiently capture particulate material that is less thanthis size. It is essential that vaccines are delivered to the Peyer's patches since they are the primary site in the gut for the induction of mucosal immune responses.

The first parameter evaluated was the viscosity of the sodium alginate. High viscosity sodium alginate could only be injected at very low rates and the low viscosity alginate did not form stable microspheres. Medium viscosity alginate (Product # A2033, Sigma) could be delivered by the Sryinge Pump and formed stable microspheres. A 1% solution of medium viscosity alginate was identified as optimal for producing microspheres less than 10 μm in diameter. A lower percentage of alginate resulted in unstable microspheres and with higher percentages of alginate the microspheres formed aggregates. Similarly, an injection pressure of 40 pounds per square inch (p.s.i.) was required to produce microspheres of optimal size. Microsphere size increased if the presures was decreased below 40 p.s.i.. Infusion rates that exceeded 2-5 ml per minute resulted in microsphere aggregation and required forces that exceeded the capacity of the Syringe pump.

Alginate microspheres are generated by aerosolizing the sodium alginate in a stream of nitrogen gas. Alginate droplets are collected in a closed vessel that contains a calcium chloride solution, which cross-links the sodium alginate so it forms stable microspheres. A 2% calcium chloride solution was optimal for the formation of microspheres with a diameter less than 10 μm. The final condition evaluated was the distance between the ultrasonic nozzle and the surface of the calcium chloride solution (aerosolization distance). A distance of less than 35-40 cm resulted in microspheres being reflected off the surface of the calcium chloride solution. An aerosolization distance greater than 40 cm resulted in microsphere aggregation prior to cross-linking in the calcium chloride solution. Flow cytometric analysis was used throughout these experiments to quantify the percentage of microspheres in each production that had a diameter less than 8-10 μm.

Alginate microspheres are stable in the calcium chloride solution but the microspheres swell and disintegrate when transferred to distilled water. We evaluated two methods to stabilize the microspheres. Two highly charged compounds, poly-L-lysine and chitosan, were evaluated. Alginate microspheres were stabilized following incubation for 30 minutes 0.05% poly-L-lysine but poly-L-lysine is very expensive (> $1.00/mg). Chitosan is much less expensive (~$1.00/g) and a 0.4% chitosan solution can also stabilized the microspheres. Thus, chitosan was considered a satisfactory

alternative to poly-L-lysine. Further *in vitro* and *in vivo* tests will be required to determine if these compounds inhibit the infectivity of the bovine adenovirus vaccine vector. After cross-linking, the microspheres can be stored for at least 2 months without a noticeable change in size or number. Thus, it should be possible to produce alginate microspheres that can be stored for prolonged periods. The stability of alginate microspheres is also being examined following freeze-drying or lyophilization. This may provide a method to extend shelf-life to months or years.

f) Characterize the interaction between alginate microspheres and the mucosal immune system.

M-cells are specialized mucosal epithelium that transport foreign material from the intestinal lumen into the Peyer's patches. Thus, they provide an interface between the intestine and the immune system and it is critical that an oral vaccine delivery system can efficiently target vaccine antigens to M-cells. To ensure that alginate microparticles provided an effective oral vaccine delivery system we developed a surgical model to directly examine the interaction between M-cells and alginate microparticles. Sterile segments of intestine were surgically prepared so that each segment contained a Peyer's patch. Individual intestinal segments were then injected with alginate microparticles that contained colloidal carbon. The encapsulation of colloidal carbon made it possible to visualize the alginate microparticles when the Peyer's patches were examined histologically. These experiments clearly demonstrated that alginate microparticles bound specifically to the the M-cells. Furthermore, following a 30-60 minute period the colloidal carbon was visible within the lymphoid follicles of the Peyer's patches. These observations clearly indicated that alginate microparticles were an effective vehicle for the delivery of vaccine antigens to the intestinal immune system.

g) Confirm that alginate microparticles delivery vaccine antigen to the Peyer's patches.

The intereaction between M-cells and alginate microparticles was further investigated by confirming that sufficent encapsulated antigen was delivered to induce a specific mucosal immune response. Surgically prepared intestinal segments were again used to compare the mucosal immune response induced by injecting either soluble protein or protein encapsulated in alginate microparticles. This comparison clearly demonstrated that injecting as much as 10 mg of soluble protein did not induce a detectable mucosal immune response. In contrast, as little as 0.5 mg of encapsulated protein induced a strong mucposal antibody response (Fig. 10). These observations confirmed that alginate micropsheres y delivered a vaccine antigen to the intestinal immune system. Furthermore, protein encapsulation in alginate microparticles resulted in more than a 20-fold increase in the efficiency of vaccine delivery.

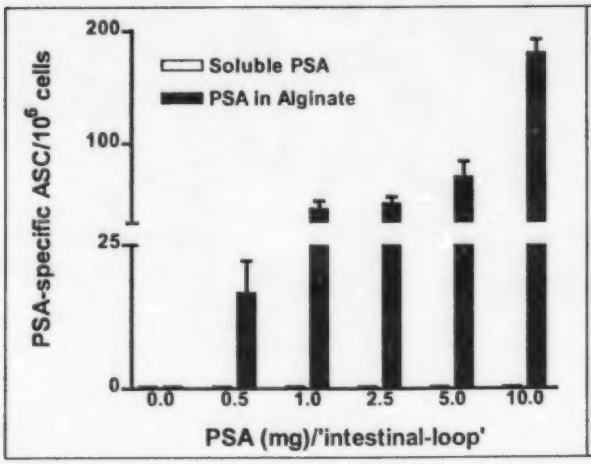


Figure 10. A specific mucosal immune response was induced by alginate encapsulated protein but not soluble protein. Porcine serum albumin (PSA) was injected into individual "intestinal'loops' of lambs as either soluble PSA or encapsulated in alginate microparticles. A specific immune response was detected by measuring the number of PSA-specific antibody secreting cells (ASC) per million jejunal Peyer's patch cells. There was a marked increase in the amplitude of the mucosdal immune response as asn increasing amount of alginate encapsulated PSA was injected into individual 'intestinal-loops.'

h) Oral Immunization with DNA vaccines.

One of our aims was to determine if multiple vaccine antigens could be encapsulated in alginate microparticles. We confirmed that both bovine adenovirus vaccine vector and protein antigens could be encapsulated in alginate microparticles and these vaccines were immunogenic in animals. In contrast, DNA vaccines encapsulated in alginate microparticles, gelatin nanospheres or viral-like particles (VP6) induced relatively weak immune responses. Experiments were then designed to evaluate DNA vaccine transfection and expression following oral immunization. One experiment involved oral immunization of fetal lambs with a DNA vaccine that was not incorporated into a delivery vehicle. This experiment clearly demonstrated that oral immunization of the fetus resulted in sufficient DNA transfection and expression to induce a strong, vaccine-specific immune response. These results were remarkable for several reasons. First, no DNA vaccine delivery vehicle was required and the DNA vaccine was delivered into the oral cavity. Secondly, all fetal lambs responded following a single oral immunization. Finally, oral immunization resulted in both a local mucosal immune response (oral cavity) and a systemic immune response. It has been very difficult to induce mucosal immune responses with DNA vaccines. These results have no immediate application to domestic animals but may be of significant value for the prevention of vertical disease transmission in humans. A better understanding of why oral DNA vaccination is effective in the fetus may provide insights into how oral immunization can be applied to older animals.

3.10.4 Conclusions:

1. A vaccine delivered to the intestine of newborn ruminants can induce immune memory that persists until the time of weaning (5-6 months of age).
2. A vaccine delivered to the intestine of newborn ruminants can induce immunity in both the intestine and the lungs.
3. These experiments indicate that developing a multivalent oral vaccine to protect against both enteric (diarrhea) and respiratory (pneumonia) disease may be appropriate. Furthermore,

including vaccine antigens for diseases that are prevalent during weaning may also be appropriate.

4. The bovine adenovirus vector is non-pathogenic in newborn ruminants, even in the absence of maternal antibody.
5. The bovine adenovirus vector is as immunogenic as the human adenovirus vector in the lamb model.
6. Subcutaneous delivery of the bovine adenovirus vector may induce both mucosal and systemic immunity. This would provide much more effective disease protection.
7. Optimal conditions were established for the consistent production of alginate microspheres.with a diameter less than 10 μm.
8. Alginate microparticles target Peyer's patch M-cells and deliver encapsulated material to the intestinal immune system
9. Alginate microspheres significantly increase the efficiency of protein antigen delivery to the mucosal immune system
10. Oral DNA immunization in fetal lambs confirmed that the immune system is fully functional in newborn ruminants and suggests that oral DNA immunization may be feasible in newborn calves

3.10.5 Information of Benefit to Producers:

This project has made substantial progress in addressing the feasibility of using oral vaccination in young calves. The following major concepts have been established.

1. The immune system in the intestine of newborn calves is fully functional and can respond to enteric vaccination.
2. The presence of maternal antibody does not interfere with the immune responses induced by enteric immunization
3. Oral immunization can induce immunity in both the intestine and the lung. Therefore, oral immunization is an appropriate approach to prevent both enteric (diarrhea) and respiratory (pneumonia) infections.
4. A single oral immunization in newborn calves can induce immune memory that persists for at least 6 months. Therefore, immunization of newborn calves may be an effective approach to prevent infectious disease during weaning.
5. Alginate microspheres can be used to delivery protein and modified-live viral vaccines to the small intestine. Thus, a simple, inexpensive oral vaccine delivery system may be available for a variety of vaccine antigens.
6. Finally, recent work suggests that it may also be possible to orally delivery DNA vaccines to newborn calves. Further work is required to fully investigate these recent observations.

In conclusion, substantial progress has been made in developing an alternative, needle-free vaccine delivery system that may have broad applicability to a wide variety of diseases.

3.11 **Project:** Vaccine formulation and delivery

3.11.1 Objectives:

a) To evaluate the adjuvanticity of liposome formulations for the delivery of subunit vaccines and to improve the immunogenicity of antigens by combining them with immunomodulators.

b) To develop methods of vaccination that are not invasive and do not require a needle. Two methods of vaccine delivery were assessed, intranasal and transdermal.

3.11.2 Background and Approach:

Vaccination can be effective method of disease prevention by stimulating the naive animal to develop protective immunity against the disease-causing organism. However, in order to optimize the response, inactivated vaccines need to be formulated with a component called an adjuvant. Only a few adjuvants are licensed for veterinary use because most adjuvants induce strong tissue reactions or create residue problems, which can affect the quality of the carcass. An effective and safe adjuvant is a critical component for the new generation of killed and recombinant protein vaccines.

We have examined the ability of liposomes as vaccine delivery systems. Liposomes are small phospholipid vesicles capable of entrapping and delivering vaccine antigens. In addition to their safety, liposomes have been shown to be able to enhance the immune response to vaccines in many animal species. We evaluated the immune response to different liposome formulations in mice.

Most infectious diseases involve colonization or invasion of the host through mucosal surfaces. Successful control of these diseases relies heavily on the development of effective strategies to stimulate mucosal immunity. Therefore, efforts to design effective methods of disease prevention should be directed to the development of initiatives to explore other non-conventional routes of delivery such as mucosal (nasal, oral, vaginal and rectal) and transdermal routes.

The main objective of this proposal was to develop and assess new adjuvant formulations that are affordable, safe and effective for use in animal vaccines. In the past we evaluated several water in oil emulsions as delivery systems. Our evaluation of new adjuvants is now being done with protective antigen gD from the BHV-1 virus. In addition, we have developed assays to measure different immune responses to BHV-1 gD. These responses contribute to protection from disease and include assays for both humoral and cell-mediated immunity.

1. The first part of our project was dedicated to formulate and characterize several different liposome preparations containing BHV-1 gD as an antigen. This characterization includes formulation of various types of liposomes to identify those with the highest yields of antigen incorporation.
2. *In vitro* and *in vivo* assays were used to assess the immunogenicity of these liposome formulations. We compared our liposome formulations to the current oil based adjuvant (VSA 3), not only in terms of the quantity but also the quality of the immune response to BHV-1 gD antigen. In addition, histological studies of the site of inoculation addressed the tissue reaction to the vaccine formulation.

3. We concentrated on designing strategies to improve liposome efficacy using cytokines (IL-4 and IL-12) and DDA. We formulated and characterized new formulations of liposomes containing the BHV-1 gD antigen with adjuvants. Since the presence of the adjuvant may interfere with the antigen incorporation, it is expected that several strategies will be needed to optimize the adjuvant-liposome formulations.
4. The *in vitro* and *in vivo* characterization was done using mice. Some of the best formulations were tested in cattle for immunogenicity.
5. Transcutaneous immunization was assessed in mice using different lipid-based delivery systems containing subunit antigens or plasmid encoded antigens.

3.11.3 Results:

a) Induction of mucosal immune responses using liposome delivery systems.

Our results demonstrated that intranasal immunization of mice with liposomes containing BHV1 gD significantly enhanced the immune responses to gD (Fig 1.).

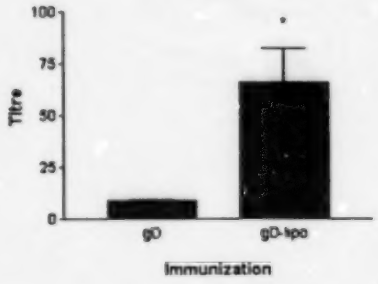


Figure 1.

One of the objectives of this project was to assess the adjuvant activity of cytokines in this model. We formulated liposomes containing IL-4 and IL-12 since these cytokines have been known to induce activation of different T helper cell activities. T helper 1 (Th1) and T helper 2 (Th2) refer to different types of immune responses. Th1 responses are mediated by IFN-γ and are important to protect the host against most viral and some bacterial diseases. In contrast, Th2 responses are mediated by secretion of cytokines such as IL-4 and are important in protecting against some bacterial diseases

We examined, therefore, the immune responses elicited by mucosal or systemic immunization of mice with liposome formulations containing bovine herpesvirus type-1 glycoprotein D (gD) together with IL-4 or IL-12. Subcutaneous immunization with liposomes containing gD and IL-12 significantly enhanced the induction of antigen-specific cellular and humoral (Fig 2) immune responses.

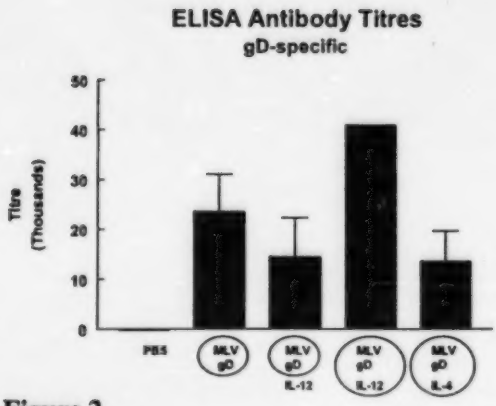


Figure 2.

These responses were characterized by the increase on IFN-γ secreting cells and the induction of gD-specific IgG2a antibodies. In contrast, incorporation of IL-4 into gD-liposomes did not enhance the humoral immune response to gD but significantly influenced the development of antigen-specific IL-4 secreting cells. Intranasal immunization with IL-12 gD-liposome formulations did not alter the induction of mucosal immune responses when compared to gD-liposomes. However, a significant increase in systemic immune responses were induced in IL-12 gD-liposome immunized mice by the intranasal route (Fig 3).

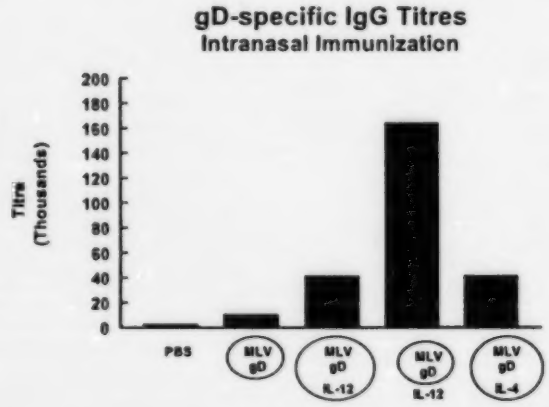


Figure 3.

While incorporation of IL-12 into the liposomes was necessary for the systemic adjuvant effect, incorporation of the cytokine was not essential for the enhancement of immune responses when the animals were immunized by the intranasal route (Fig 2 and 3). These findings indicate that the requirements for cytokines to influence immune responses to a vaccine antigen differ between systemic and mucosal routes.

Establishment of a mouse model to assess immunogenicity of bacterial antigens.

Current work in our laboratory is been designed to evaluate other vaccine antigens using liposomes as adjuvants. This experiments include characterization of liposome formulation using *Haemophilus somnus* antigens (Tbp1, Tbp2, Lpp and HSP). We will be testing the ability of these formulations to induce mucosal and systemic immune responses in mice.

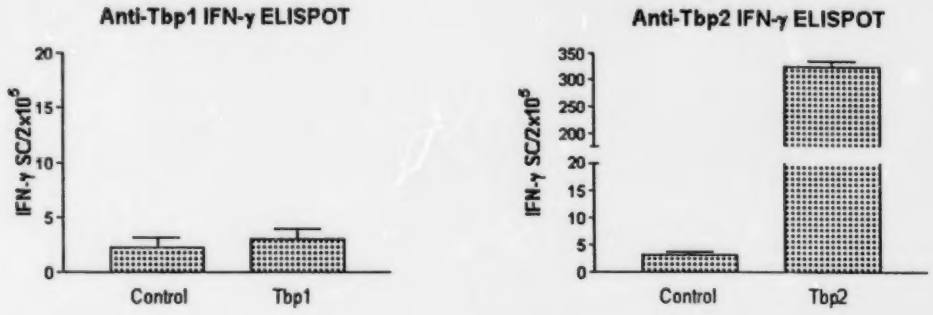


Figure 4. Interferon gamma secreting cells (IFN-γ SC) in mice immunized with Tbp1 and Tbp2. Mice were immunized with 20 μg of Tbp1 or Tbp2 and boosted three weeks later. Spleen cells were isolated one week after the boost and cultured in the presence of either Tbp1 or Tbp2.

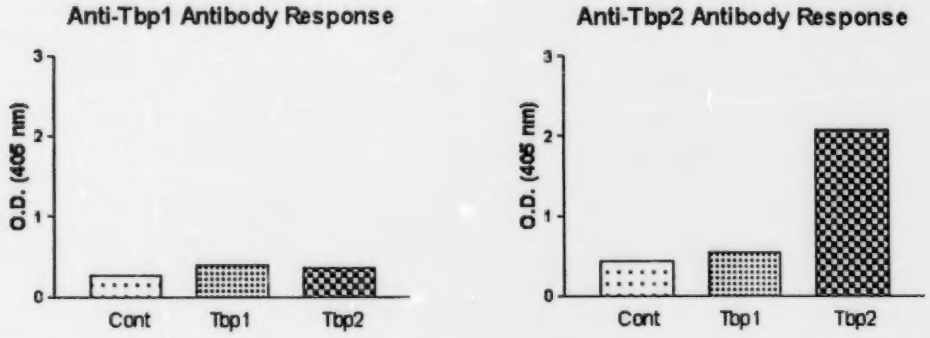


Figure 5. Antibody response in mice immunized with Tbp1 and Tbp2 antigens. Animals were immunized as in Fig. 1. The antibody levels in serum indicate that Immunization with Tbp1 did not induce Tbp1 antibodies. In contrast, immunization with Tbp2 induced good antibody levels in mice.

We have established the immunological assays to evaluate immune responses to Tbp1 and Tbp2 in mice. This battery of assays will allow us to carry out more specific experiments with the aim of evaluating the efficacy of different adjuvants and delivery systems using this vaccine antigens. All this preliminary work is important before we can take this vaccine into cattle.

These preliminary results showed that Tbp1 does not induce cellular (IFN-γ) or humoral (antibody) responses. In contrast, Tbp2-immunized mice showed high levels of both cellular and

humoral responses. More experiments are needed to explore the mechanisms responsible for this discrepancy.

Mucosal immunization of cattle using a liposome based vaccine.

An experiment was designed to assess the ability of gD-liposome formulations to induce mucosal immunity in cattle. Groups of 10 animals were immunized intranasally with formulations containing placebo, gD alone (25 μg/animal) and gD in liposomes (25 μg/animal). The results of this experiment are shown in Fig. 3 and 4.

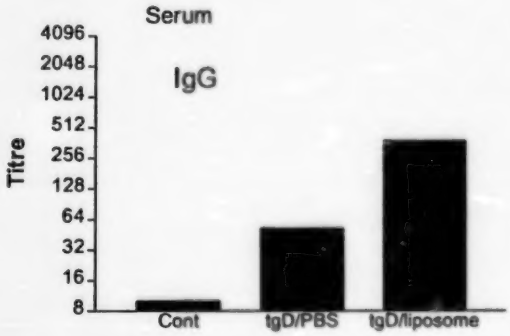


Figure 6. Antibody response to gD in serum of immunized cattle. Animals were immunized intnasally and boosted 21 days later. Serum samples were collected 10 days after the second immunization.

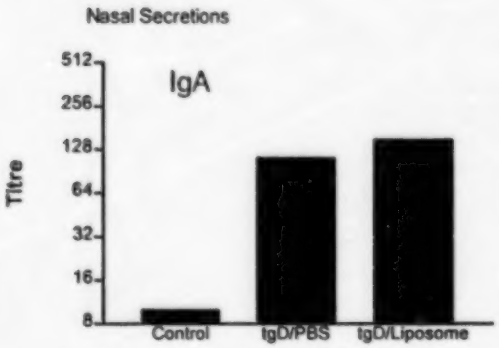


Figure 7. Antibody responses in the mucosal secretions following intranasal immunization. Animals were immunized as in Fig.3. The gD-specific IgA titres were measured in nasal secretions.

Intranasal vaccination with liposome formulations induced significantly higher gD-specific IgG responses in serum. Interestingly, the gD-specific IgA responses in the mucosal secretions were similar between the groups immunized with gD or with gD in liposomes. These results indicate that induction of systemic (serum) responses was enhanced by liposome delivery, whereas induction of local antibody response was not. One explanation for these results is that the gD molecule is capable of binding to cells (it is a viral protein involved in cell adhesion) and therefore it is likely that gD could by itself bind to epithelial cells.

Intranasal Immunization of Cattle with Pasteurella haemolytica (Leukotoxin, Ltx) Vaccine.

We demonstrated that intranasal administration of bovine herpes virus type-1 (BHV-1) vaccine (gD) formulated in liposomes was capable of inducing serum and mucosal specific-antibodies. These results led us to examine whether bacterial antigens such as the Ltx from *Pasteurella haemolytica* could induce similar immune responses.

Intranasal immunization of cattle with Ltx did not induce significant enhancement of specific antibodies when compared to the control group (fig 3). Interestingly, all animals including

the control group, had high anti-Ltx antibody titres (8,000). These high background may have limited the ability of our vaccine protocol to enhance immune responses to Ltx.

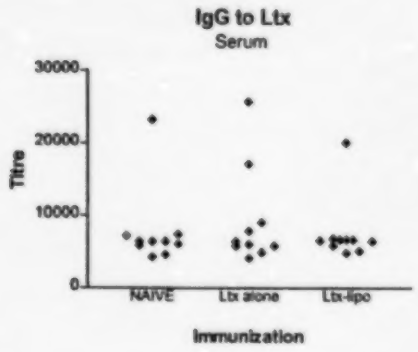


Figure 8. Antibody responses in cattle following intranasal immunization with saline (naïve), Ltx alone and Ltx formulated in liposomes (Ltx-lipo). Each dot represents an individual animal. Note the high background level of anti-Ltx antibodies in the control group (mean of 7796).

Intranasal Immunization of Sheep with Leukotoxin (Ltx) and Cholera toxin (CT) antigens.

The following series of experiments were designed to evaluate the mucosal and systemic immune responses after intranasal immunization of sheep with two bacterial antigens, Ltx and CT. There are two important reasons to use sheep instead of cattle, one is the economical and the other is that both species have immune systems that are functionally and developmentally identical.

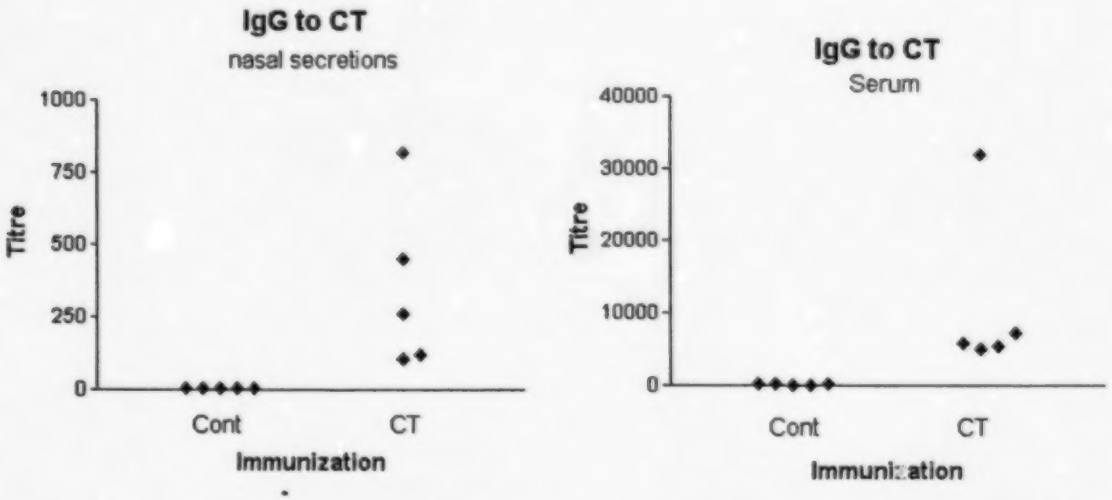


Figure 9. Antibody responses to CT in the nasal secretions and in serum of sheep following intranasal immunization with CT formulated in liposomes. Each point represents individual animals.

Intranasal immunization with CT induced significant increase in mucosal (nasal secretions) and systemic (serum) specific IgG antibodies (fig.4). Due to technical problems we have been unable to measure CT-specific IgA. However, we have contacted researches in Australia and they have agreed to provide us with the reagents to measure sheep IgA. We expect that for the next report we will have this information.

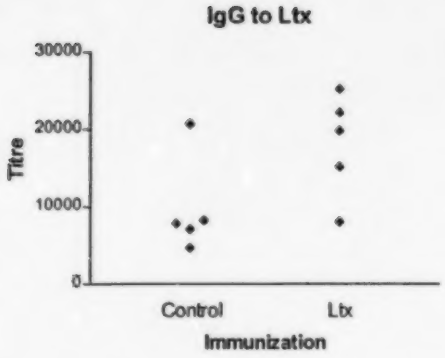


Figure 10. Antibody responses in serum following intranasal immunization of sheep with Ltx. Immunized animals received 100 μg of Ltx formulated in liposomes. Each dot represents individual animals. High background levels of anti-Ltx are present in non-immunized control group.

Intranasal immunization of sheep with Ltx did not significantly enhanced the specific-antibody levels in serum (fig.5). Although the response was not statistically significant there was a trend that immunized animals had higher levels of Ltx-specific antibodies.

In conclusion, the results presented in this report illustrate that intranasal immunization of cattle and sheep can induce specific immune responses against the vaccine antigen. However, we also demonstrated that control non-immunized animals (cattle and sheep) contained high levels of anti-Ltx antibodies and that intranasal immunization with Ltx failed to enhance immune responses to this antigen.

Intranasal Immunization of Cattle with Liposome Formulations Containing gD and Quil-A as an Adjuvant.

We have previously shown that immunization of cattle with gD antigen formulated in liposomes induced mucosal immune responses against gD. As a second step in this immunization approach we designed an experiment to assess the effect of Quil-A in enhancing the mucosal immune response using liposomes as delivery systems. The experiment was carried with calves from the Western Beef Development Center at the Termuende Farm.

The experimental design consisted of 5 groups of 6 animals each. The treatments consisted in: gD alone; gD in liposomes; gD + Quil-A and gD + Quil-A in liposomes. The negative control group received saline. All animals were immunized intranasally and 3 weeks later were boosted with the same formulation and by the same route. Ten days after the boost all animals were bled and the nasal secretions collected to measure the levels of gD-specific antibodies (IgA and IgG).

In this experiment the liposome-gD formulations did not induce a systemic or mucosal immune response. In addition, Quil-A had no effect in enhancing immune responses to gD. At the present time we do not have an explanation for the negative results of this experiments. We are currently assessing the feasibility of the intranasal route to induce immune responses in cattle. The methods to deliver the vaccine may have to be redesigned to allow better administration of the formulations.

Assessment of Transdermal Delivery of Antigens for Induction of Immune Responses in Mice.

One of the main objectives for this past reporting period was the evaluation of transdermal immunization using a novel lipid-based biphasic delivery system.

We have initiated these experiments in mice and the results have been positive. We are currently designing new approaches to improve the responses and to transfer the technology to large animals.

Pasteurella haemolytica is a gram negative bacteria commonly isolated from the lungs of cattle with pneumonic pasteurelosis. Leukotoxin (Ltx) an exotoxin produced by *Pasteurella haemolytica* has been identified as a potential virulence factor, the recombinant protein produced in *E. coli* is currently used to immunize cattle to protect against fibrinous pneumonia. In the present study we used Ltx and hen egg lysozyme as model antigens to investigate the ability of transdermal delivery of vaccine antigens to induce humoral (antibody) and cellular (antigen-specific proliferation and IL-4 secretion) responses in mice.

Assessment of immune responses induced by transdermal delivery of Ltx.

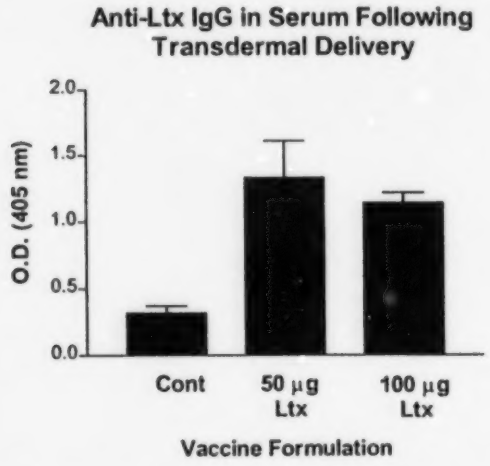


Figure 11. Serum antibody responses following transdermal immunization with Ltx liposome patches. Mice were immunized by the transdermal route with 50 µg or 100 µg of Ltx, control animals received patches containing liposomes alone. The immunization was repeated on day 21 and the animals were bled 10 days later. Ltx-specific antibodies in serum were analyzed by ELISA and are expressed as optical density (O.D.) 405 nm at a serum dilution of 1:160.

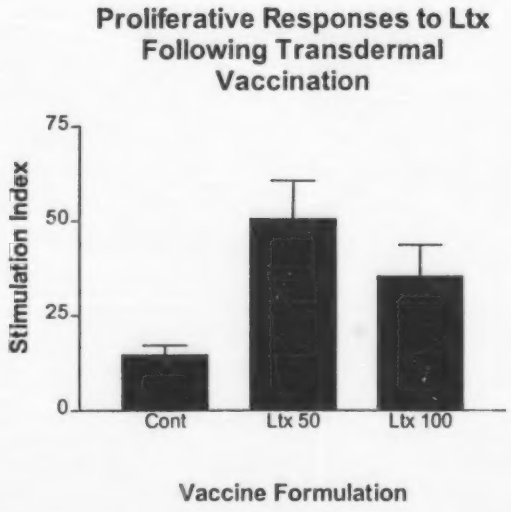


Figure 12. Proliferative responses of spleen cells following *in vitro* stimulation with 25 μg/ml of Ltx. Spleen cells were obtained from mice immunized with 50 μg or 100 μg of Ltx, vaccination was repeated on day 21 and the animals were sacrificed 10 days later. Results are expressed as mean stimulation index of 5 mice and the bars indicate the standard deviation.

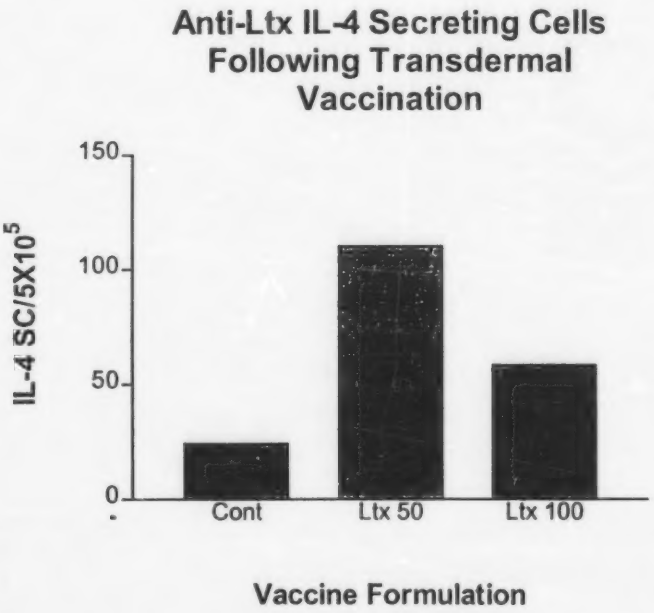


Figure 13. Number of IL-4-secreting cells following *in vitro* stimulation of spleen cells with Ltx. Mice were immunized and boosted two weeks later. Spleen cells were harvested 10 days after the second immunization and cultured in the presence of Ltx for 48 hours prior to detection of cytokine. Results are expressed as the mean of IL-4-secreting cells/5X10^5 cells of triplicate wells of pooled spleen cells from 4 mice.

Effect of transdermal delivery of Ltx on the induction of cell-mediated immunity measured by ELISOT assay for IL-4 and IFN-γ secreting cells

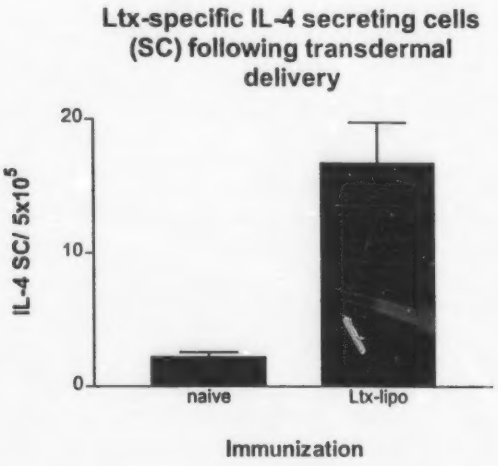


Figure 14. To assess the type (Th1 vs Th2) and magnitude of cell-mediated immune responses induced by the transdermal administration of Ltx, the IL-4 and IFN-γ secreting cells were determined in spleens cells from immunized animals. The results demonstrated that the T-cell response was characterized by secretion of IL-4. No IFN-γ specific responses were observed.

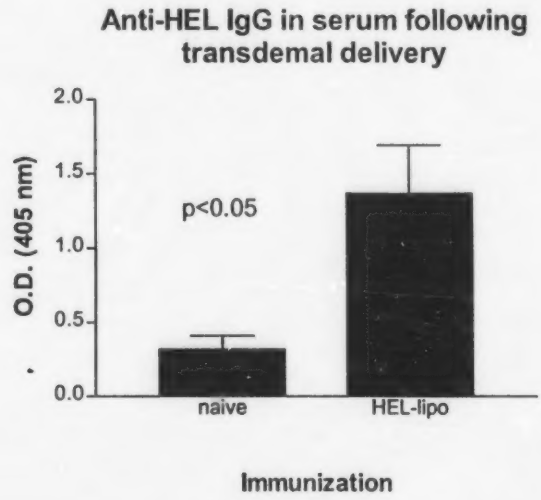


Figure 15. Anti-HEL IgG response following transdermal administration of antigen. HEL alone or formulated in biphasic liposomes. The antibody levels were measured using an ELISA and the results expressed as the optical density of a 1:640 serum dilution of individual mice.

Induction of HEL-specific antibody response in serum by transdermal immunization with HEL in different Biphasic delivery formulations

We have previously shown that Lkt formulated in a Biphasix delivery system was capable of inducing humoral and cellular immune responses when delivered by the transdermal route. This experiment was designed to assess whether induction of immune responses by the transdermal route could be achieved with other proteins, and whether different formulations of the Biphasix delivery system influenced these responses. Hen egg lysozyme has been used extensively as a model antigen in the evaluation of immune responses in mice. Therefore, we selected HEL to evaluate the immune responses elicited following transdermal immunization. Topical administration of 50 μg of HEL formulated in various Biphasix compositions showed that formulation of the delivery system had an impact on the level of humoral and cell-mediated immune responses induced. All animals immunized with Biphasix delivery system developed HEL-specific IgG titres in the serum (Fig. 6). However, there were significant differences between different formulations in their ability to induce HEL-specific IgG responses. Mice immunized with formulation V induced the highest levels of antigen-specific IgG in serum. The anti-HEL IgG response was characterized primarily by IgG1 subclass in all groups (Table 1). *In vitro* antigen stimulation of cells isolated from spleen and lymph nodes of immunized mice showed a predominant IL-4 response over IFN-γ (Fig. 7). Again, mice immunized with formulation V produced a higher proportion of IL-4 secreting cells when compared to the response of mice immunized with other formulations.

To determine whether the route of immunization influenced the immune response to HEL, mice were immunized with 50 μg of HEL formulated in the same delivery system by the subcutaneous and transdermal routes. Our observations indicated that the HEL-specific antibody response (Fig. 8) elicited by both routes of immunization were similar.

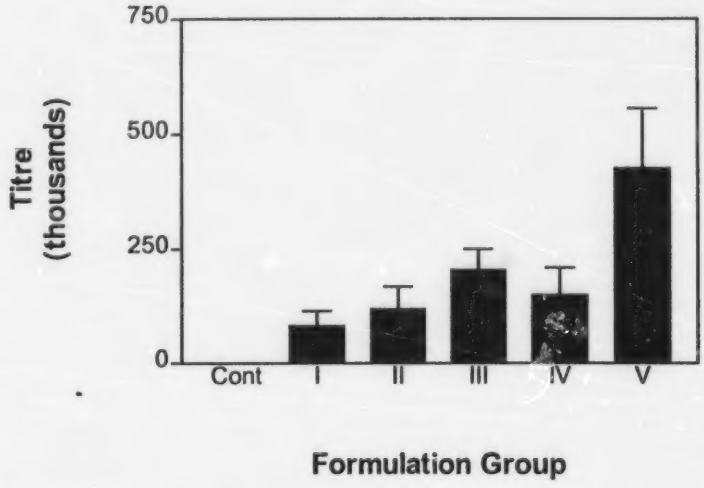


Figure 16. HEL-specific IgG response in serum of transdermally immunized mice with 50 μg of HEL formulated in different Biphasix delivery systems. Data represent mean titres ± SEM of five individual mice.

Induction of HEL-specific IFN-γ and IL-4 secreting cells following transdermal immunization with different Biphasix formulations.

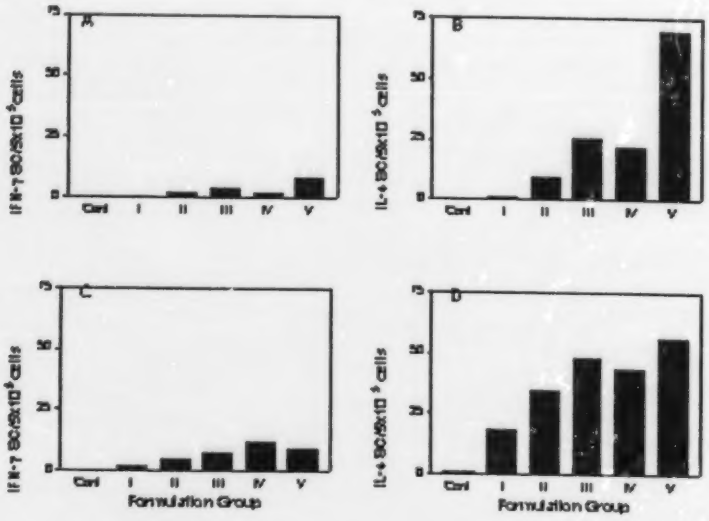


Figure 17. HEL-specific IL-4 and IFN-γ secretion of spleen cells and draining lymph nodes cells following topical immunization with 50 μg of HEL formulated in different Biphasix delivery systems. Cells were cultured in the presence of 20 μg/ml of HEL and the frequency of IL-4 and IFN-γ-secreting cells in the lymph nodes (a and b) and spleen (c and d) was assessed by ELISPOT assay. Data represent the mean cytokine-secreting cells of pooled samples from five individual mice.

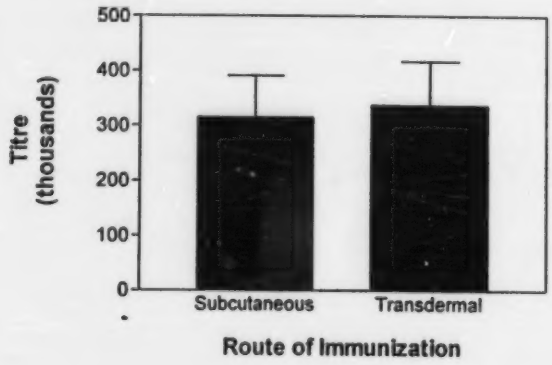


Figure 18. Comparison between subcutaneous and transdermal routes in the induction of HEL-specific IgG in serum. Mice were immunized subcutaneously or transdermally with 50 μg of HEL formulated in the same Biphasix formulation and the levels of anti-HEL were determined in serum using an ELISA. Data represent mean titres ± SEM of five individual mice.

Table 1. HEL-specific IgG isotypes in serum following transdermal immunization with different Biphasix formulations.

Formulation	HEL-Specific IgG Isotype (GM)*			
	IgG1	IgG2a	IgG2b	IgG3
Control	<40	<40	<40	<40
I	402000	365	445	749
II	77040	1352	395	1681
III	728900	460	471	5964
IV	260400	490	1505	454
V	1335000	2036	3450	4379

* Titres are expressed as the geometric mean of 5 individual mice.

Assess the potential of transdermal vaccines carrying a plasmid DNA encoding a vaccine antigen to induce immune responses in cattle and sheep.

The development of delivery systems that can mediate transfection in vivo have not been fully characterized. Therefore, the testing of these formulations in cattle or sheep has been delayed. We carried out experiments to characterize these formulations in vivo. Five groups of BALB/c mice (three mice/group), were immunized either with 100 μg of gD encoding plasmid in a lipid-based delivery vehicle or with 1 μg gD encoding plasmid using a gene gun. Immunizations were administered 3 times at 2 week intervals. One day before each immunization mice were shaved and the skin left to recover before immunization. Two weeks after the last immunization gD specific humoral and cellular immune responses were tested using ELISA and ELISPOT respectively.

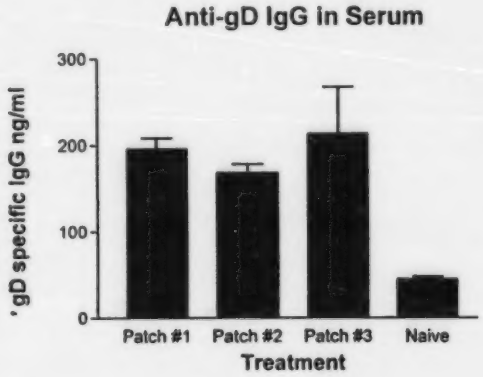


Figure 19. This graph demonstrates the serum anti-gD IgG titres induced by transdermal delivery of plasmid encoding gD. ELISAs were done on individual mouse serum for total gD specific IgG. Although gD specific antibody was detected in all the immunized mice, gene gun immunized mice had much greater gD specific antibody 5000 ng/ml compared to 200 ng/ml obtained by mice immunized using a patch.

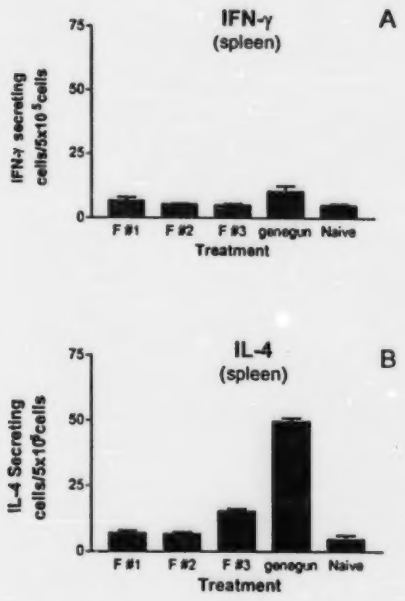


Figure 20. This graph demonstrates the cytokine secretion of cells from transdermally immunized mice with a plasmid encoding gD. IFN-γ (A) and IL-4 (B) secreting cells were done using individual spleen cells. Cells were stimulated with 0.4 μg/ml of gD.

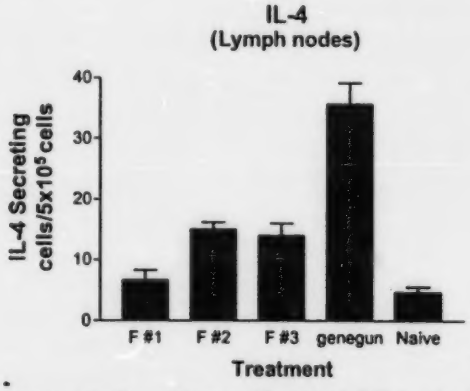


Figure 21. This graph demonstrates the gD-specific IL-4 SC in draining lymph nodes of immunized mice. IL-4 SC were found in gene gun immunized mice as well as in some groups immunized with the lipid-based formulations. No IFN-γ SC were detected in any group.

Assess the potential of transdermal vaccines (protein and DNA) to induce protection against BHV-1 infection in cattle.

The assessment of transdermal vaccines in cattle to protect against disease has not been done due to the fact that the immune responses induced in large animals have been variable and low. Since challenge experiments in cattle are very expensive, we have decided to postpone these experiments until new formulations, more suitable for the penetration of the skin of cattle, are developed.

Assess the potential of transdermal vaccines carrying a protein antigen to induce mucosal and systemic immunity in cattle and sheep.

Following the successful induction of immune responses in mice using transdermal vaccines, our next objective for this period was to evaluate the effect of topical application of vaccines in sheep and cattle. We conducted a number of experiments to evaluate the thickness of the skin in different parts of the animal and to determine whether immune cells (Langerhans cells) were stimulated by the application of lipid-based formulations when applied to the skin.

Our results demonstrated that the permeability barrier of the skin in cattle and sheep can not be penetrated by the formulations used in mice. Therefore, we assessed whether potent enhancers of permeability could disrupt this barrier and allowed the induction of immune responses. The results of these experiments showed that some animals (20%) developed immune responses to the vaccine antigen, suggesting that the skin of sheep is equipped with immune cells capable of mediating the induction of immune responses.

At present the barrier properties of sheep and cattle skin are not well understood. Reports in the literature suggested that drugs are much more likely to traverse sheep and cattle skins by way of skin appendages (i.e. hair follicles, sweat gland, and ducts) rather than through the cells of the stratum corneum. However, little is know about how drugs penetrate the skin of sheep and cattle. It is possible that one of the reasons why some pour-on formulations are very successful in penetrating the skin of sheep and cattle is because they use strong solvents. Strong organic solvents can dissolve the lipid-rich barrier of the skin and allow the penetration of drugs. Unfortunately, due to trait secret, the composition of most of the pour-on formulations have not been fully disclosed.

We have realized that in order to manipulate the permeability barrier of the skin of cattle we need to have a better understanding of the characteristics of the skin in terms of structure, immune competence and permeability. For these reasons we initiated some experiments with the aim of determining the structure and immune characteristics of sheep and cattle skin. We established immunohistological assays to evaluate the presence of immune cells. These assays will allow us to evaluate the changes that occur in the immune cells of the skin following the application of different formulations. Because these assays can be done in the laboratory using skin form dead animals, this preliminary screening will be much less expensive than experiments using live animals.

We have evaluated the effect of two immunomodulators (IL-12 and IFN-α).

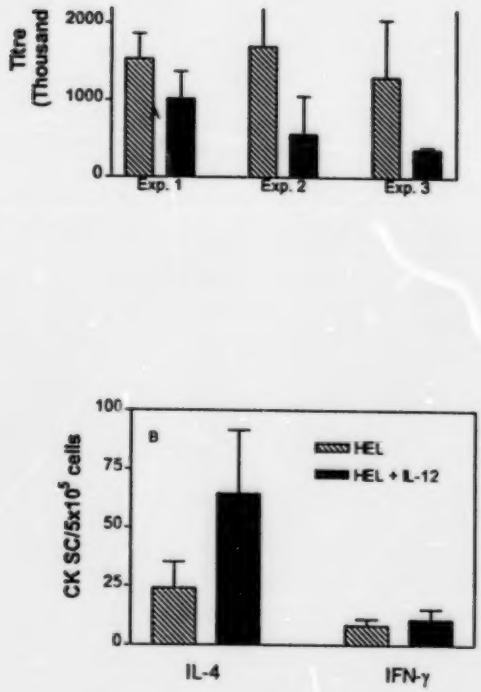


Figure 22. To examine the effect of IL-12, a potent vaccine modulator, on the induction of humoral and cellular immune responses in mice. Co-administration of IL-12 and the antigen hen egg lysozyme (HEL) decreased the antibody response compared to the immune responses induced by administration of HEL alone (A). The cellular responses were not significantly changed (B). These results were different from our previous experiment using IL-12 as an immunomodulator for systemic and mucosal vaccines (Baca-Estrada et. al. 1997 and Baca-Estrada et. al. 1999). However, these experiments indicate that it is possible to modulate the immune responses by incorporating an immunomodulator into the vaccine formulation.

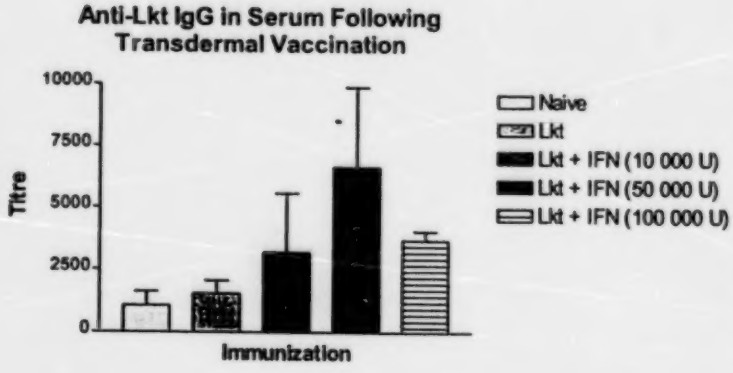


Figure 23. Effect of IFN-α on the humoral immune response to Lkt following transdermal delivery. Mice were immunized with 10 μg of Lkt alone or together with different doses of IFN-α. The serum of immunized mice was analyzed by ELISA for the presence of anti-Lkt IgG. The results are expressed as the mean titre ± SD from 5 mice/group.

Assess the potential of transdermal vaccines (protein and DNA) to induce protection against BHV-1 infection in cattle.

The assessment of transdermal vaccines in cattle to protect against disease has not been done due to the fact that the immune responses induced in large animals have been variable and low. Since challenge experiments in cattle are very expensive, we have decided to postpone these experiments until new formulations, more suitable for the penetration of the skin of cattle, are developed.

We have clearly demonstrated that lipid-based delivery systems can induce immune responses to protein vaccines when applied by the transdermal route in mice. We have also extended our observations indicate that these responses can be modulated by the addition of cytokines. We have initiated experiments to evaluate this vaccination approach in cattle by investigating the characteristics of their skin and to evaluate the incorporation of strong permiation enhancers and compounds that will allow a better penetration of the vaccines through the skin.

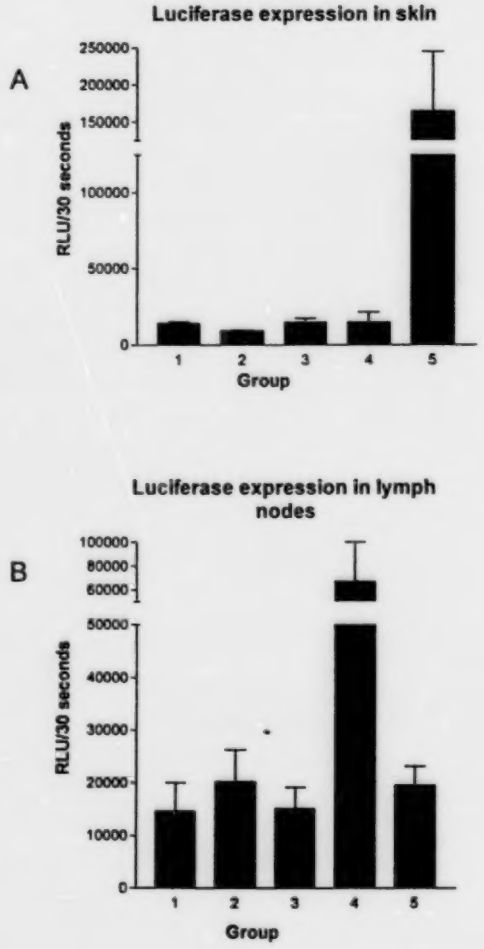


Figure 24. *In vivo* expression of luciferase following topical (groups 1-4) or intradermal injection (group 5) of plasmid encoding luciferase (pMAS-luc). Mice (5/group) received 100 μg topically or 10 μg intradermally of pMAS-luc as indicated above. Forty eight hours later they were euthanized and skin (A) or draining lymph nodes (B) were obtained and processed as indicated in Table 6. The results are expressed as relative light units per 30 seconds.

Having established these methods, we are now in the position of assessing important questions regarding protein and DNA delivery by lipid-based formulations. The results of these experiments will be very important for the design of vaccine transdermal delivery systems that are effective in stimulating immune responses in large animals (pigs and cattle).

Evaluation of Immune Responses and Protection Against Actinobacillus preuropneumoniae by intranasal Immunization with Oml-A Formulated in a lipid-based formulation.

Previous work from our Institute has demonstrated that intramuscular immunization of pigs with the outer membrane lipoprotein (OmlA) of *Actinobacillus pleuropneumoniae* induces protective immune responses against respiratory infection with *A. pleuropneumoniae* (1). Our preliminary experiments using this infection model suggested that intranasal immunization with OmlA formulated in Biphasix™ delivery system induced protective immunity in the respiratory tract. This protection was superior to that induced with immunization with antigen alone. Therefore, in order to evaluate whether the protection conferred by immunization with OmlA formulated in Biphasix™ is significant, we will design an experiment with more animals per group.

The objective of this proposal is to assess the effectiveness of intranasal immunization with Biphasix™ vaccine delivery system in protecting pigs against a respiratory infection.

Groups of 6-8 pigs (5-6 weeks old) will be intranasally immunized on days o, 10 and 20 with 100 mg of OmlA alone or formulated in either formulation # 1-7-3 or #1-7-4. On day 29 serum and nasal washes were collected to measure anti-OmlA IgG and IgA. On day 30 all animals were challenged with *A. pleuropneumoniae* serotype 1.Clinical evaluation was done by a veterinarian and tissue samples will be collected for bacteriological evaluation (the presence of *A. pleuropneumoniae*).

Induction of OmlA-specific systemic and mucosal immune responses

Intranasal immunization of pigs with Biphasix formulations induced significantly higher OmlA-specific IgG antibodies in serum when compared to immunization with OmlA in PBS (Fig 1). In addition, intranasal immunization of pigs with Biphasix/OmlA formulations stimulated mucosal immune responses as measured by the presence of OmlA-spcific IgA and IgG antibodies in nasal secretions (Fig. 2).

Protection against Actinobacillus pleuropneumoniae intranasal challenge

Immunized pigs were challenge with *Actinobacillus preuropneumoniae* using an aerosol chamber as described by Gerlach, G. F. *et al.* (1). Animals were monitored 3 times a day for clinical signs of pneumonia and rectal temperatures were recorded. Clinical scores were based on the severity of respiratory distress, the degree of general clinical illness and depression. End-points were in accordance with the guidelines of the Canadian Council on Animal Care. All surviving mice were killed on day 7 post-infection. The number of pigs surviving the challenge (survivors/total) were as follows: Naïve group 2/6; OmlA/PBS 2/8; OmlA/1-7-3 2/8; OmlA/1-7-4 4/8.

3.11.4 Conclusions:

The data presented in this section highlights the major results in the area of vaccine formulation. Development of novel vaccine adjuvants to replace mineral oil is an important area of research for the Vaccine Formulation and Delivery Team at VIDO. It is for this reason that we focused much of our own research into assessing a more suitable, biodegradable oil such as liposomes or cytokine molecules as potential adjuvants.

Most infectious diseases involve colonization or invasion of the host through mucosal surfaces. Induction of mucosal immunity as a first line of defense against most pathogens is crucial. However, since it is well recognized that systemic immunization is not always efficient in inducing mucosal immunity, immunostimulation of mucosal sites is best achieved by administration of antigens at mucosal surfaces. Liposomes have not only been proven effective in delivering vaccine antigens by the systemic route but are also capable to inducing mucosal immunity when orally or intranasally administered. We evaluated whether our liposome vaccine preparations could stimulate development of immunity following systemic and mucosal (intranasal or oral) immunization.

- Our results in mice suggest that intranasal immunization with gD in liposomes is more efficient in inducing mucosal responses than the vaccine antigen alone. We are now in the process of taking this approach to immunized cattle.
- Transdermal delivery of a bacterial antigens such as Ltx induces antigen-specific humoral and cellular responses. The cellular response is characterized by the predominant induction of antigen specific IL-4 over IFN-γ. The proportion IL-4 secreting cells was higher in the draining lymph nodes than in the spleen of immunized mice.
- Topical application of HEL formulated in biphasic liposomes induced significant anti-HEL IgG.
- Transdermal delivery of a bacterial antigens such as Ltx induces antigen-specific humoral and cellular responses. The cellular response is characterized by the predominant induction of antigen specific IL-4 over IFN-γ. The proportion IL-4 secreting cells was higher in the draining lymph nodes than in the spleen of immunized mice.
- Topical application of HEL formulated in biphasic liposomes induced significant anti-HEL IgG.
- Transdermal immunization of mice with HEL formulated in a Biphasix delivery system induces cellular and humoral immune responses. Our results demonstrated that the immune response was of a Th2 type characterized by the predominant induction of antigen-specific IgG1 antibody in serum and IL-4-secreting cells in the spleen and lymph nodes.
- The induction of immune responses to the antigen are influenced by the formulation of the delivery system. Our results suggest that optimal Biphasix formulations need to be established for each vaccine antigen to induce the most effective immune response.
- Transdermal delivery of HEL in Biphasix formulations induces similar levels of specific IgG antibodies in serum than subcutaneous immunization with the same formulation.
- Formulations capable of stimulating immune responses in mice following topical application of plasmid DNA have been developed.

- We have clearly demonstrated that lipid-based delivery systems can induce immune responses to DNA vaccines when applied by the transdermal route in mice.
- Methods to evaluate the penetration of vaccines through the skin and activation of immune cells (Langerhans cells) have been developed.
- Immunomodulation of immune responses induced by transdermal vaccines (protein) have been observed using IL-12 and IFN-α.
- We have established immunohistochemistry techniques for the assessment of Langerhans cells in skin of mice, pigs and cattle.
- We have established a model to assess *in vivo* transfection following topical administration of plasmids.
- Biphasix™ formulations significantly enhanced stimulation of OmlA-specific IgG responses in serum when compared immunization with OmlA in PBS.
- Intranasal immunization of pigs with OmlA induced both IgG and IgA in nasal secretions. Although the OmlA-specific IgA titres were higher in animals immunized with Biphasix™ formulations, these responses were not significantly different from the OmlA/PBS immunized group.

3.11.5 Information of Benefit to Producers:

The potential of developing cost effective vaccine delivery systems provides an opportunity to enhance disease control and safety over currently available vaccines. Preventing disease with better vaccines should improve farm efficiency and profits as well as resulting in consumer savings. Added benefits may be incurred through the reduction of carcass trim and hide damage due to reactive vaccines and excessive antibiotic treatment. VIDO continues to update producers groups on the results of this research on an ongoing basis.

1. RESEARCH/LICENSING AGREEMENTS – INTELLECTUAL PROPERTY

Research/Licensing Agreements

January 1, 2000. A License Agreement between the University of British Columbia, Alberta Research Council Inc. with the objective of furthering research into the development of a bovine vaccine against Enterohemorrhagic *E. coli.*

December 1, 2000. A Services Agreement was signed with Genetronics, Inc. whereby VIDO performs services related to swine research.

April 1, 2000. Merck & Co. and VIDO have signed a study agreement relating to the creation of a bovine adenovirus vector expressing an HIV gag protein.

March 31, 2000. A research agreement was signed with Boehringer Ingelheim Vetmedica, Inc. to work on the development of a vaccine for Bovine Mastitis.

January 1, 2000. Pecura, a division of Qiagen (Canada) Inc. has provided support for the study of DNA vaccines for BRSV and BVD and immunostimulatory oligonucleotides.
September 7, 1999. A collaborative agreement between Dr. Janice Berg of Lakeside Farm Industries Ltd. to study "Water quality Management for Pre-Harvest Control of *Escherichia coli* O157:H7".

July 26, 1999. Bayer AG agreed to fund research on the "Construction and testing of a BVDV-specific BAV3 vector".

December 23, 1998. A collaborative agreement was signed with the Commonwealth Scientific and Industrial Research Organization to collaborate on the development of bovine and porcine adenoviruses as vectors for the delivery of antigens active against diseases in cattle and pigs.

October 6, 1998. Agriculture and Agi-Food Canada Inc. and VIDO have agreed to collaborate on the "Development of Plant-Derived Porcine Parvovirus Empty Capsids for Oral Vaccination of Swine".

October 1, 1998. A research agreement was signed with Qiagen GmbH relating to DNA vaccines for bovine respiratory syncytical virus and bovine virus diarrhea. Qiagen GmbH has been granted an option to obtain an exclusive world-wide license with the right to sub-license the Intellectual Property resulting from the Research Program.

September 24, 1998. Pharmaderm Laboratories Ltd. and VIDO as sole or joint owners of intellectual property have signed a royalty sharing agreement relating to vaccine delivery technology.

July 23, 1998. A Research/Option Agreement was signed between VIDO, the University of Guelph, University Technologies International Inc., the Canadian Microbiology Consortium Inc. and Bayer Corporation to conduct a project which would involve development of the technology related to TbpA, TbpB, and LppB of *Pasteurella haemolytica* or *Haemophilus somnus*, for vaccines against infections caused by *Pasteurella haemolytica* or *Haemophilus somnus* in cattle. During the term of the agreement, Bayer has an exclusive option to negotiate an exclusive worldwide license to use and commercialize the above technology and any resulting Intellectual Property.

July 13, 1998. A Research and Marketing Agreement was signed with Vetrepharm Canada Inc. which grants Vetrepharm the use of VP8 rotavirus protein, a coronavirus protein designated S2 and related technology.

July 13, 1998. BTG International Inc. has signed a commercialization agreement for rotavirus carrier technologies entitling them to an exclusive, world-wide license.

May 22, 1998. A Research Agreement was signed with Bayer A.G. to conduct a project entitled "Adenovirus Vectors for Vaccine Delivery". Upon completion of the project, Bayer A.G. has the option to continue the project or to license the resulting technology.

May 19, 1998. A Research Agreement funding a project related to Post-Weaning Multisystemic Wasting Syndrome and its possible causative Porcine Circovirus was signed with Boehringer Ingelheim/Nobl Laboratories, Inc. An option to license the resulting technology is open following the completion of the Project.

April 3, 1998. A Letter of Intent was signed between Dr. W. Lubitz, Bayer A.G. and VIDO for the Delivery of DNA Vaccines by Bacterial Ghosts.

January 1, 1998. A Research Collaboration agreement dealing with Bovine Adenovirus 3 Based Vectors was signed with Transgene.

March 13, 1997. A licensing agreement was signed with Laboratoire Service International, Lissieu, France granting LSI an exclusive license in France to develop and market ELISA kits using reagents developed at VIDO.

February 2, 1996. A Research and Marketing Agreement was signed with the National Veterinary Institute granting an exclusive license to test Haptoglobin reagents for the development of a Bovine Haptoglobin test.

Patents Issued on Which VIDO Staff are Inventors

United States Patent No. 6,086,890
Title: Bovine adenovirus expression vector system.
Date: July 11, 2000
Inventors: Mittal, S.K., Graham, F.L., Prevec, L., Babiuk, L.A.

European Patent No. 061020
Title: Vaccines for *Actinobacillus pleuropneumoniae.*
Date: February 9, 2000
Inventors: Potter, A.A., Gerlach, G. Willson, P. Rossi-Campos, A.

United States Patent #6,022,960
Title: GNRH-Leukotoxin chimeras.
Date: February 8, 2000
Inventors: Potter, A.A., and Manns, J.G.

United States Patent #6,001,591
Title: Recombinant bovine adenoviruses.
Date: December 14, 1999
Inventors: Mittal, S.K., Graham, F.L., Prevec, L., and Babiuk, L.A.

United States Patent #5,985,289
Title: *Haemophilus somnus* outer membrane protein extract enriched with iron-regulated proteins.
Date: November 16, 1999
Inventors: Potter, A.A. and Harland, R.J.

United States Patent #5,969,126
Title: GNRH-Leukotoxin chimeras.
Date: October 19, 1999
Inventors: Potter, A.A. and Manns, J.G

Switzerland Patent #0,618,814
Title: Recombinant bovine herpesvirus type 1 polypeptides and vaccines.
Date: October 15, 1999
Inventors: Babiuk, L.A., van Drunen Little-van den Hurk, S., Zamb, T., and Fitzpatrick, D.

Germany Patent No. P 692 28 944.5-08
Title: Recombinant bovine herpesvirus type 1 polypeptides and vaccines.
Date: September 27, 1999
Inventors: Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick, D.

Swiss Patent based on European Patent No. 0 618 814
Title: Recombinant bovine herpesvirus type 1 polypeptides and vaccines.
Date: September 27, 1999
Inventors: Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick, D.

United States Patent No. 5,951.988
Title: Adjuvant formulation with enhanced immunogenic activity, and related compositions and methods.
Date: September 14, 1999

Inventors: van Drunnen Littel-van den Hurk, S., Zamb, T. and Redmond, M.

New Zealand Patent No. 308991
Title: CAMP factor of *Streptococcus uberis*.
Date: August 12, 1999.
Inventors: Potter, A.A., Jiang, M

United States Patent No. 5,891,677
Title: *Actinobacillus pleuropneumoniae* outer membrane lipoprotein A and uses thereof.
Date: April 6, 1999
Inventors: Gerlach, G.F., Willson, P.J., Rossi-Campos, A., Potter, A.A.

United States Patent No. 5,879,895
Title: Recombinant bovine herpesvirus type 1 polypeptides and immunoassays.
Date: March 9, 1999
Inventors: Babiuk, L., van den Hurk, S., Zamb, T. and Fitzpatrick, D.

United States Patent No. 5,876,725
Title: *Actinobacillus pleuropneumoniae* transferring-binding protein vaccines and uses thereof.
Date: March 2, 1999
Inventors: Potter, A. A., Gerlach, G. F., Willson, P. J., Rossi-Campos, A.

United States Patent No. 5,871,750
Title: Leukotoxin vaccine compositions and uses thereof.
Date: February 16, 1999.
Inventors: Potter, A.A.

United States Patent No. 5,863,543
Title: CAMP factor of *Streptococcus uberis*.
Date: January 26, 1999
Inventors: Potter, A.A., Jiang, M. and MacLachlan, P.R.

United States Patent No. 5,858,989
Title: Vaccines comprising nucleotide sequences encoding bovine herpesvirus type 1, G1, GIII and GIV.
Date: January 12, 1999
Inventors: Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick D.

European Patent No. EP 0 635 055 B1
Title: *Haemophilus somnus* immunogenic proteins.
Date: December 23, 1998.
Inventors: Potter, A.A., Pontarollo, R.A., Pfeiffer, C.G., Theisen, M., Harland, R.J. and Rioux, C.

United States Patent No. 5,849,531
Title: Compositions and treatments for pneumonia in animals.
Date: December 15, 1998
Inventors: Potter, A.A.

United States Patent No. 5,837,268
Title: GnRH-leukotoxin chimeras.
Date: November 17, 1998
Inventors: Potter, A.A. and Manns, J.G.

Netherlands Patent No. 0,659,086
Title: Novel bacterial vaccines using vaccine strains of pathogenic bacteria.
Date: November 11, 1998.
Inventors: Allan, B. and Potter, A.A.

United States Patent No. 5,820,868
Title: Recombinant protein production in bovine adenovirus expression vector system.
Date: October 13, 1998
Inventors: Mittal, S.K., Graham, F.L., Prevec, L. and Babiuk, L.A.

United States Patent No. 5,801,018
Title: Vaccines for Actinobacillus pleuropneumoniae.
Date: September 1, 1998
Inventors: Potter, A.A., Gerlach, G.F, Willson, P.J. and Rossi-Campos, A.

United States Patent No. 5,747,309
Title: Bacterial vaccines using vaccine strains of pathogenic bacteria.
Date: May 5, 1998
Inventors: Allan, B.J. and Potter, A.A.

United States Patent No. 5,723,129
Title: GnRH-leukotoxin Chimeras.
Date: March 3, 1998
Inventors: Potter, A.A. and Manns, J.G.

Australian Patent No. 683,221
Title: Novel bacterial vaccines using vaccine strains of pathogenic bacteria.
Date: February 26, 1998
Inventors: Allan, B.J. and Potter, A.A.

United States Patent No. 5,708,155
Title: Enhanced immunogenicity using leukotoxin chimeras.
Date: January 13, 1998
Inventors: Potter, A.A., Redmond, M.J. and Huw, P.A.H.

United States Patent No 5,672,350

Title: Recombinant bovine coronavirus E2 and E3 polypeptides and vaccines.
Date: September 30, 1997
Inventors: Parker, M.D., Cox, G.J., and Babiuk, L.A.

European Patent No 0527724
Title: Compositions and treatments for pneumonia in animals.
Date: August 27, 1997
Inventors: Acres, S.D., Babiuk, L.A., Potter, A.A., and Lawman, MJ.P.

European Patent No 0652957
Title: Assembled viral particles and their use in a vaccine to rotaviral disease.
Date: May 7, 1997
Inventors: Redmond, M.J., Ijaz, M.K., and Parker, M.D.

United States Patent No 5,594,107
Title: Chimeric protein comprising an RTX-family cytotoxin and interferon-2 or interferon.
Date: January 14, 1997
Inventors: Andrew A. Potter, Manuel Campos, and Hugh Hughes

United States Patent No 5,585,264
Title: Nucleotide sequences encoding bovine herpesvirus type-1 gI, gIII, and gIV polypeptides.
Date: December 17, 1996
Inventors: Babiuk, L.A., van den Hurk, S., Zamb, T., and Fitzpatrick, D.R.

United States Patent No 5,521,072
Title: *Actinobacillus pleuropneumoniae* transferrin binding proteins and uses thereof.
Date: May 28, 1996
Inventors: Potter, A.A., Gerlach, G.F., Willson, P.J. and Rossi-Campos, A.

5. PERSONNEL AND EQUIPMENT

No equipment was purchased with this funding. The following individuals received salary support (April 1, 1996 – February 28, 2001). Funding from this grant is used to cover a portion of the entire operations of VIDO and therefore each individual receiving support from this grant spends 100% of his/her time on the operations relating to the grant.

Person	Salary
Andrew Potter	$102,522
Robert Brownlie	2,857
Trent Watts	32,944
Dean Tatlow	9,607
Yuanhao Li	707
J.B. Lee	432
Brenda Allan	225,633
Dale Godson	91,797
Sylvia van den Hurk	186,740
Chengru Zhu	602
Sam Attah-Poku	23,051
Barry Carroll	113,217
Monique Hambermehl	21,459
Jamie Mamer	50,183
Carolyn Olson	62,998
Brock Evans	53,815
Elizabeth Umelo	7,764
Cam Raggo	9,585
Susantha Gomis	47,674
Heidi Seida	272
Heather Haywood	3,589
Rachelle Buchanan	5,980
Ralph Braun	30,152
Tricia Dublainco	4,019
Jodi Ell	8,988
Adam Hueser	1,481
Jason Hueser	514
Cindy Toy	3,374
Don Wilson	66,619
Stuart Bond	12,542
Tracey Bruneau	21,506
Philip Griebel	170,200
Sanipa Suradhat	3,565
Maria Baca-Estrada	31,402
George Mutwiri	52,801
Phil Willson	35,412
Brad Wildeman	1,500
Don Winslow	2,497
	$1,500,000

6. COMMUNICATIONS

Research Publications in Scientific Journals

Baca-Estrada, M.E., Foldvari, M., Ewen, C., Badea, I., and Babiuk, L.A. 2000. Effects of IL-12 on immune responses induced by transcutaneous immunization with antigens formulated in a novel lipid-based biphasic delivery system. Vaccine 18: 1847-1954.

Baca-Estrada-M., Foldvari, ,M., Snider, M., Harding, K., Kounikakis, B., Babiuk, L.A., and Griebel, P. 2000. Intranasal immunization with liposome formulated *Yersinia pestis* vaccine enhances mucosal immune responses. Vaccine 18: 2203-2211.

Baxi, M.K., D. Dereget, J. Robertson, L.A. Babiuk, T. Schlapp, and S.K. Tikoo. 2000. Recombinant bovine adenovirus type 3 expressing bovine viral diarrhea virus glycoprotein E2 induces an immune response in cotton rats. Virology 278:234-243.

Braun,R.P., Babiuk, L.A., Loehr, B.I. and van Drunen Littel-van den Hurk, S. 1999. Particle-mediated DNA immunization of cattle confers long-lasting immunity against bovine herpesvirus-1 Virology 265*: 46-56.*

Dosman, J.A., Senthilselvan, A., Kirychuk, S.P., Lemay, S., Barber, E.M., Willson, P., Cormier, Y., Hurst, T.S. 2000. Positive human health effects of wearing a respiratory in a swine barn. Chest 118: 852-860.

Gerdts, V., Babiuk, LA., van Drunen Little-van den Hurk, S., and Griebel, P.J. 2000. Fetal immunization by a DNA vaccine delivered orally into the amniotic fluid. Nature Medicine 6: 929-932.

Gibb, D.J., Schwartzkopf-Genswein, K.S., Stookey, J.M., McKinnon, J.J., Godson, D.L., Wiedmeier, R.D., and McAllister, T.A. 2000. Effect of a trainer cow on health, behavior, and performance of newly weaned beef calves. J Anim Sci 78: 1716-25.

Gomis, M.S, Gomis, A.I.U., Horadagoda, N.U., Wijewardene, T.G., Allan, B.J., Potter, A.A. 2000. Prevalence and description of cellulitis and other *Escherichia coli* disease syndromes in broilers in Sri Lanka. Trop. Anim., Hlth. Prod. 32: 341-351.

Griebel, P.J., Beskorwayne, T., Godson, D.L., Popowych, Y., and Hein, W. 2000. Cloning non-transformed sheep B cells. J Immunol Methods 237: 19-28.

Lewis, P.J., van Drunen Littel-van den Hurk, S., and Babiuk, L.A. 1999. Effect of antigen compartmentalization on the immune responses to a gD DNA vaccine. J. Virol. 73:10214-1023.

Lewis, P.J., van Drunen Littel-van den Hurk, S., and Babiuk, L.A. 1999. Development of immune responses in the presence of passive antibody. J. Gen . Virol. 80: 2829-2837.

Liu, Q., Wang, L., Willson, P., and Babiuk, L. 2000. Quantitive, competitive PCR analysis of porcine circovirus DNA in serum from pigs with post-weaning multisystemic wasting syndrome. J. Clin. Microbiol. 38: 3473-3477.

Loehr, B.I., Willson, P., Babiuk, L.A., and van Drunen Littel-van den Hurk, S. 2000. Gene gun mediated DNA immunization primes development of mucosal immunity against bovine herpesvirus-1 in cattle. J. Virol. 74: 6077-6086.

Mutwiri, G., Bateman, C., Baca-Estrada, M.E., Snider, M., and Griebel, P.J. 2000. Immunocompetence of gut-associated lymphoid tissue in newborn lambs. Vaccine 19: 1284-1293.

Olfert, E.D. and Godson, D.L. 2000. Humane endpoints for infectious disease animal models. Institute for Laboratory Animal Research Journal 41: 99-104.

Raggo, C., Habermehl, M., Babiuk, L.A., Griebel, P.J. 2000. *In vivo* effects of a recombinant bovine herpesvirus-1 vector expressing bovine interferon-gamma. J. Gen. Virol. 81: 2665-2673.

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